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RAPD - PCR analysis of l-asparaginase producing marine bacterial species

K.Vinoth Kumar and S.Paranjothi

Department of BioTechnology, M.G.R College, Dr., M.G.R Nagar, Hosur - 635109, India.

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ABSTRACT

The marine biosphere is one of the richest habitats of microorganisms. Marine microbes particularly bacteria are considered for their secondary metabolites and enzymes with novel properties. The Marine Soil Microbial isolates were screened for potential producers of L-asparagines using a phenol red indicator and growth medium. The isolates were characterized by biochemical tests and found to belong to *Bacillus* sp. We performed random amplification of polymorphic DNA (RAPD) analysis on five strain of *Bacillus* sp. Random primers were used for the PCR. Electrophoresis on denaturing acrylamide gels improved RAPD reproducibility and increased the band number. The primer of OPU series gave reproducible results and band profiles.

Introduction

L-asparaginase is known for its potent ability to be used as a therapeutic agent for the treatment of leukemia since many years. Microbial L-asparaginase has attracted considerable attention since the demonstration that L-asparaginase from E.coli has anti tumor activity (Broome et al., 1961). L – asparaginase is now known to be a potent antineoplastic agent in animals and has given complete remission in some human leukemia's found L-asparaginase to be responsible for the antitumor activity of guinea pig serum (Joseph et al., 1968; Kidd et al., 1953). Subsequently, it was identified as an effective antitumor agent in human clinical trials, and today it is regarded as one of the useful components of the antitumor therapy. Some of the L-asparaginase enzyme derived from E.coli was also proved as an effective antitumor activity similar to guinea pig serum (Mashburn et al., 1964). This event opened up the possibility of large scale production of the enzyme for ultimate clinical trials. As a result, now a day's more attention has been given to isolate the L-asparaginase enzymes by microorganisms. Different microorganisms were used for the screening, some of the Pseudomonas species were also proved for the production of L-asparaginase enzyme (Subha et al., 1995).

L-asparaginase (L-asparagine aminohydrolase, EC 3.5.1.1), the enzyme which converts L-asparagine to L-aspartic acid and ammonia, has been used as a chemotherapeutic agent. It has received increased attention in recent years for its ant carcinogenic potential (Fisher et al., 2002; Manna et al., 1995). The clinical action of this enzyme is attributed to the reduction of L- asparagines; tumor cells unable to synthesize this aminoacid are selectively killed by L-asparagines deprivation. Though several L-asparaginases of bacterial origin have been developed and their potential usage in clinical trials have been studied to prevent the progress of L-asparagines- dependent tumors, mainly lymph sarcomas, the success hitherto has been rather limited, and most of the treatments must be interrupted due to severe side effects and immunological reactions in the patients.

Recent development in DNA technology have made it possible to uncover a large number of genetic polymorphism at the DNA sequence level, and to use them as makers for the observed the phenotypic variability. Random amplification of polymorphic DNA by the polymerase chain reaction is a means of detecting polymorphisms for genetic mapping and strain identification. The method has considerable appeal because it is generally faster, less expansive than any previous method for detecting DNA sequences variation and does not require prior sequences information (Welsh et al., 1990). The fact that RAPDs survey multiple loci in genomic makes the method attractive for analysis of genetic distance and phylogeny reconstruction (Williams et al., 1990). Perhaps the main reason for the success of RAPD analysis is the gain of a larger number of markers that require small amount of DNA without the requirement for cloning, sequencing or any other for form of molecular characterization of the genome of the species in question. It is a powerful tool in DNA fingerprint analysis of various animal species, gene mapping studies to population analysis and identification of genetic variability of different microbial species (Bardakci et al., 2001). The objective if the present study was to access molecular variation among Bacillus sp and to determine the level of genetic similarity among them. We performed random amplification of polymorphic DNA (RAPD) analysis on five strain of Bacillus sp. Random primers were used for the PCR (Ron et al., 1997). Electrophoresis on denaturing acrylamide gels improved RAPD reproducibility and increased the band number. The primer OPU serious gave reproducibility result and the band profile were used to create the phylogenetic tree by using UPGMA.

Materials and Methods

Sample collection from marine environment

The marine sediment samples were collected from different area of Bhairavapalam – coringa, Kakinada (Andhra Pradesh), Yanam (Pondicherry). The sample ware collected in sterile plastic bottle.

Tele:

E-mail addresses: vino.indya@yahoo.com

Selective Isolation of L-Asparaginase Producing Marine Bacteria

About 5 gm of sample was taken and suspended in 95 ml of sterile distilled water in a 250 ml conical flask and kept in a rotary shaker with 120 rpm for 30 minutes for the thorough mixing of the sediment sample. About 1 ml of mixed sediment suspension from conical flask was transferred in to 9 ml of sterile distilled water. The sample was serially diluted up to 10-5 dilutions. Sea water agar plates were prepared and used for the isolation of L-Asparaginase producing marine bacteria. Components of SWA(gram/liter) includes sea water agar part – A peptic digest of animal tissue: 5g, yeast extract : 5g, beef extract: 3g, agar :15g and sea water agar part B : Sodium Chloride: 24g, Potassium : 0.70g, MgCl26H20 : 5.30g, MgSO47H20: 7g, Calcium Chloride :0.10g, and PH :7.5 Himedia (Prakasham et al., 2010).

After sterilization, the plates were prepared and 0.1 ml of aliquot from 10-3 to 10-5 dilutions were taken and spreader on sea water agar medium by using sterile L-rod. Plating was done in triplicates. All the plates were incubated at 28°C for 1 week. One Un inoculated plate was kept as control. All the plates were observed from second day of incubation (figure 1).

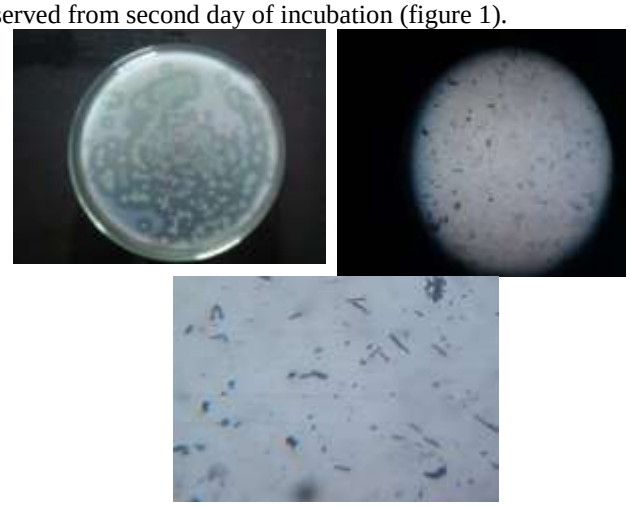

Figure 1: Smooth, off white, circular colonies, Gram positive Bacillus (rod) cells.

Screening of L-Asparaginase Producing Marine Bacteria

During incubation, morphologically different bacterial colonies were selected. To obtain pure culture, the selected colonies were streaked on (Modified M - 9). Agar-based modified M9 medium (composition (g/l): KH 2 PO4 : 2.0g ,L-asparagines : 6.0g , MgSO4 .7H 2O : 1.0g , CaCl2 .2H 2O : 1.0g , Glucose : 3.0 g , and Agar : 20.0 g) supplemented with phenol red (few drops) as indicator medium agar by phase streaking and incubated at 37 °C in Bacterial incubator for 5 days (Gulati et al., 1997). After incubation, the red colour producing plates were selected and sub cultured on nutrient agar slant and stored at 4ºC until further study (figure 2).

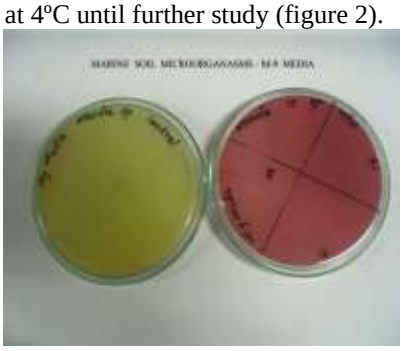

Figure 2: Selection of L - Asparaginase Producing Marine Bacteria on M – 9 Media and Biochemical test

Molecular Biology

Genomic DNA Isolation

The Bacillus cultures were inoculated in Luria-Bertani broth and incubated at 37□c for 48 hours. The inoculated culture was centrifuged at 6000rpm for 10minutes.Bacillus cells were collected in the form of pellets at the bottom and discard the supernatant. Repeat the above step to give an increased concentration of cells. Re suspend the pellet in 1ml of lysis buffer. Incubate at 45□C in boiling water bath for 10 minutes. Add 1ml of phenol: chloroform mixture (1:1) and centrifuge at 10,000rpm for 10 minutes. To the supernatant (upper aqueous layer) add equal volume of chloroform: isoamyl alcohol mixture (24:1) and 1/20th volume of 3M sodium acetate and centrifuge at 10,000 rpm for 10 minutes. To the upper aqueous layer add double volume of chilled ethanol and incubate at -20□C for 20minutes. Then Centrifuge at 12,000rpm for 10minutes and air-dry the pellet. Dissolve the DNA pellet in 20-50□l TE buffer.

Evaluation of Quality and Quantity of genomic DNA

The quality of isolated genomic DNA was evaluated by agarose gel electrophoresis using 0.8% agarose gel and 1 X TBE buffer. The concentration of DNA was also checked by UV spectrophotometer taking optical density (OD) at 260 and 280 nm.

Quantitative analysis of DNA

The purity and quantity of the isolated DNA was determined by Nanodrop spectrophotometry (ND 1000). The extinction ratio (260/280 nm) absorbance ratio for all the samples ranged from 1.7 to 1.9 indicating high quality of DNA (Figure 3).

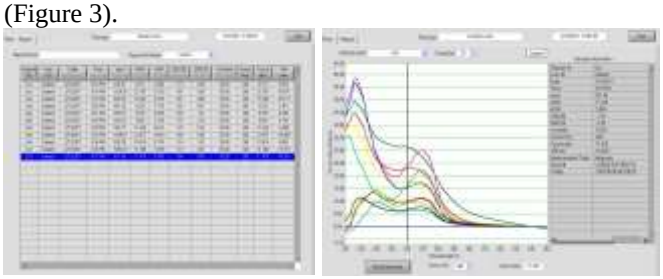

Figure 3: Reports of quantitative Analysis of DNA samples

RAPD –PCR Optimization

The PCR reaction mixture was prepared based on the (Table 1). The amplification was carried out for 40 cycles with initial denaturation at 950C for 5 min, second denaturation for 1 min at 940C, annealing at 360C for 1 min and extension for 2 min at 720C and final extinction at 720C for 5 min . All the amplified products were resolved by electrophoresis in 1.5% of agarose gel using 1x TAE buffer at 50volt for 2 ½ hours . A 100 base pair ladder was included as molecular size marker. Gels were visualized by staining with Ethidium bromide (1µl/10ml) and banding patterns were photographed over UV light (Figure 4). Five random primers were used for RAPD PCR Optimization such as OPU 2(CTGAGGTCTC), OPU

5(TTGGCGGCCT), OPU (ACCTTTGCGG), OPU 7(CCTGCTCATC) and OPU 8(GGCGAAGGTT).

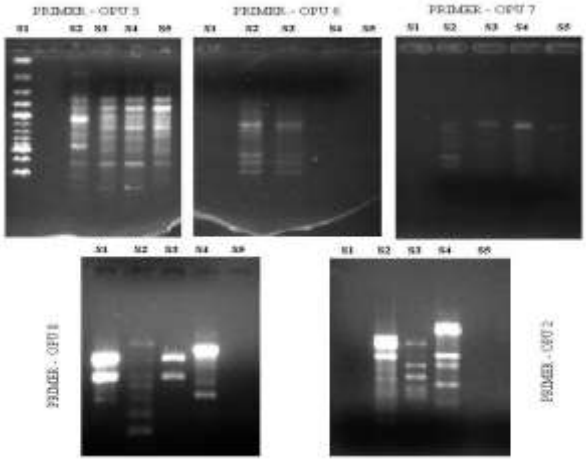

Figure 4: The PCR products obtained were analyzed on 1.5% agarose gels

Data analysis

Only clear bands of RAPD product on agaroge gels were scored. Genetic similarity index was calculated on the basis of band frequencies. Mean average percentage different (MAPD) was determined by the formula of Phylogenetic variation were determined by converting RAPD data into a frequency similarity and analyzed by Unweighted Pair Group Method with Arithmetic mean (UPGMA) cluster analysis to produce a phylogenetic tree (Gilbert et al., 1990).

Result and Discussion

The RAPD technique was used to find out the genetic variability of among the marine Bacillus sp. The present study deal with the isolation of DNA using Phenol:Choloroform extraction method from different Bacillus sp. RAPD - PCR was performed using five different primers of OPU series of OPU 2, OPU 5, OPU 6, OPU 7and OPU 8 supplied by Chromous Biotech, Bangalore.

The PCR protocol as adapted in the study results in the reproducible patent of amplification using specific combination of accession and primes. All the primers used in this study displayed reproducible, scorable and clear bands and they were analyzed. The image profile of banding patent was recorded and molecular weight of each band was determined by Alpha Innotech Software. The banding patterns were scored 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 all the individual or accession. The most intense monomorphic band from each accession with each primer was used as reference to calibrate different lanes for the amount of DNA present. When there were no monomorphic bands, the bands with maximum frequency in each accession were used to calibration. In each lane, bands were scored present if their intensity was at least 10% of the monomorphic reference bands within the same lane. Using Dice coefficient, a similarity matrix involving 5 accession was generated with NTSYS –pc. A dendrogram was constructed using the unweighted pair Group method with Arithmetic Average of all five samples. Maximum bands were observed with primer OPU (25678). A sum total of 20 bands were amplified with respect to all five primers. About 8 bands (29.52%) wee polymorphic. The number of monomorphic bands was 12 (70.47%). The dendrogram figure based on S1 showed distinct separation of the collected accession through monomorphically they were similar and inseparable the dendrogram separated the five accession of same genus with OPU series primers into two major groups.

Among the two major clustars , the accession belonging to the upper cluster (UC) 1,3,4,5,6,9,10,17,19 and 20.While accession belonging to the lower cluster were 2,7,8,11,12,13,14,15 and 16. Further, accession of the UC were grouped into two major subclusters USC 1 and USC2, The upper sub cluster 1 (USC1) had a solitary collection from 1, 3, 17, 18, 19 and 20. While the upper sub cluster 2 (USC2) comprise of 10, 4 (87%) similarity and 1, 5 shows (70%) similarity and the dice coefficient value 10, 4 (0.40) and 1, 5 (0.29).

USC 1 is further divided into Trunk I and trunk II , The Trunk 1 is having accession 17,19 and 20 the similarity value for 17,19 (75%) and Trunk II having accession 3,18 (75%) the dice coefficient value (0.33) . The Dice coefficient value between trunk I and trunk II is (0.14) and the similarity between them 64%, The Lower cluster shows all the accession are 100% similarity(Figure 5 and Figure 6).

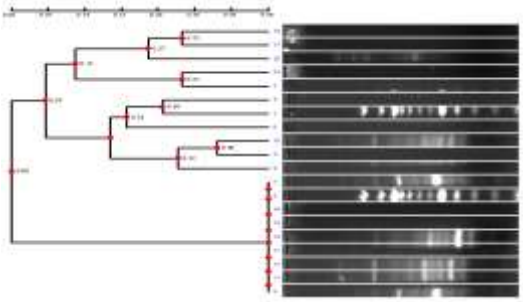

Figure 5: RAPD of 5 strains of Marine *Bacillus sp* with OPU Series of Five primes

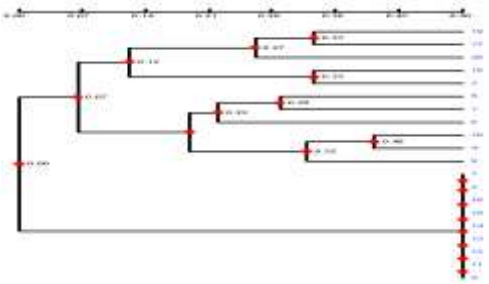

Figure 6: RAPD of 5 strains of Marine Bacillus sp with OPU Series of Five primes

Conclusion

The marine biosphere is one of the richest habitats of microorganisms. Marine microbes particularly bacteria and considered for their secondary metabolites and enzymes with novel properties. They are having different medical property. The preliminary attempet is to distinguish the genetic diversity among the five different marine bacterial sp producing L – Asparaginase species using RAPD studies performed. The L – Asparaginase has some medical properties. It act as a Anti cancer agent, known for its potent ability to be used as a therapeutic agent for the treatment of leukemia and anti-neoplastic agent in animals. The marine sediment samples were collected from different area of Bhairavapalam – coringa , Kakinada , Yanam and Pondicherry. And it was stored at -200 C in deep freezer. The DNA was isolated from each species of marine bacterial sp using lysis buffer method. The isolation extract was analysed both qualitatively and quantitatively. Qualitatively it was done by using 0.8% Agarose gel electrophoresis (AGE). It examines the intensity of the isolated DNA and checks the contamination. Quantitatively it was

analysed by using a NANO-Drop which works under the principle of UV- Visible Spectrophotometer. Thus the concentration of the profile was recorded. Then the RAPD – PCR analysis was done using the Unweighed Pair Group Method with Arithmetical (UPGMA) based on the similarity index. Further result from RAPD is used to construct gene mapping and the development of specific molecular marker for this desired species.

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References

1. Broome, J.D., Evidence that the L-asparaginase activity of guinea pig serum is responsible for its antilymphoma effects. Nature 1961; 191:1114-1115.
2. Bardakci F, Random amplified polymorphic DNA (RAPD) markers, Turk J Bio 200l; 25 185-196.
3. Fisher SH, Wray Jr LV Bacillus subtilis 168 contains two differentially regulated genes encoding L-asparaginase. J. Bacteriol 2002; 184: 2148-2154.
4. Gulati, R., Saxena, R. K. and Gupta, R. A rapid plate assay for screening L-asparaginase producing micro-organisms. Lett. Applied Microbiol 1997; 24: 23-26.
5. Gilbert D A, Lehman N, O Brien S J & Wayne R K, Genetic fingerprinting reflects population differentiation in the California channel island fox, Nature (Lond) 1990; 344 764-766.
6. Joseph Roberts., Gene Burson. And Joseph M.Hill. New Procedures for purification of L-asparaginase with high yield from Escherichia coli. J. Bacteriol 1986; 95: 2117-2123.
7. Kidd, J. G., Regression of transplanted lymphomas induced in vivo by means of normal guinea pig serum. I. Course of transplanted cancers of various kinds in mice and rats given pig serum, horse serum or rabbit serum. J. Expt. Med 1953; 98:565-582.
8. Mashburn, L.T. and Writson, J. C., Tumor inhibitory effect of L-asparaginase from Ecoli. Arch Biochem. Biophysics 1964; 105:451-452.
9. Manna S, Sinha A, Sadhukan R, Chakrabarty SL .Purification,characterization and antitumor activity of L-asparaginase isolated from Pseudomonas stutzeri MB-405. Curr Microbiol1995; 30: 291-298.
10. Prakasham RS, Hymavathi M, Subba Rao Ch, Arepalli SK, Venkateswara Rao J, Kavin Kennady P, Nasaruddin K, Vijayakumar JB, Sarma PN .Evaluation of antineoplastic activity of extracellular asparaginase produced by isolated Bacillus circulans. Appl. Biochem. Biotechnol 2010; 160: 72-80.
11. Ron, S., Ronimus. Lynne, E., Parker.. The utilization of RAPD-PCR for identifying thermophilic and mesophilic Bacillus species, Federation of European Microbiological Societies1997; 88: 46-49.
12. Subha, M., Amaresh, S., Ramakrishna, S. and Chakrabarty, S. L., Purification, characterization and antitumor activity of Lasparaginase isolated from Pseudomonas stutzeri MB-405.Curr. Microbiol 1995; 30:291-298.
13. Welsh, J. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res 1990; 18: 7213-7218.
14. Williams, J. G. K. DNA Polymorphisms amplified by arbitrary primers are useful as genetic marker, Nucleic Acids Res1990; 18: 6531-6535.
15. Williams. J. G. K., Hanafey. M. K., Rafalski. J. A., and Tingey, S. V. Genetic analysis using RAPD Markers. Methods Enzymol 1993; 218: 704-740.

Table: 1 PCR reaction mixture condition

S. No	Sample Compound	Stock Conc.	Working Conc.	Working Volume
1.	DNA	78.2ng/µl	75ng/µl	1µl
2.	Primers (5´-3´)			
	OPU 2(CTGAGGTCTC)	……..	300pm	1µl
	OPU 5(TTGGCGGCCT)	……..	300pm	1µl
	OPU 6(ACCTTTGCGG)	……..	300pm	1µl
	OPU 7(CCTGCTCATC)	…….	300pm	1µl
	OPU 8(GGCGAAGGT)	……..	300pm	1µl
3.	Tag Polymerase	3000 U	3U	1µl
4.	*dNTP´s*	10Mm	800µm	2µl
5.	*Assay Buffer*	10X	1X	2.5µl
6.	Distilled Water	_	_	17.7µl
			TOTAL	25 µl