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Mutation Detection in Bacillus Amyloliquefaciens by Random Amplified Polymorphic DNA – (RAPD) PCR

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ABSTRACT

The random amplified polymorphic DNA (RAPD) PCR technique has been used to detect mutation in Bacillus amyloliquefaciens by Ultraviolet treatment. Six different primers (decamer) were used for amplification. Initially, there were tested 6 decamer primers from (Sigma genosys) which didn't produce any amplification, excepting 1 of them VAA 18. VAA 18 (decamer primer) amplified with DNA of Bacillus amyloliquefaciens and showed polymorphism. After data analysis, 15 out of 9 bands were polymorphic. The result obtained from primer VAA 18 showed changes in band pattern which reflected DNA alternations or mutation. After the dendrogram analysis indicated the fact that the genetic similarity showed by all four mutant strains but not with wild strain. On the basis of dendrogram mutant strains had some genetic changes or mutation against the wild strain.

Keywords: ***Bacillus amyloliquefaciens, RAPD, Primer, Agarose gel electrophoresis, DNA isolation, CTAB method, polymorphism***

Introduction

The polymerase chain reaction (PCR) has become one of the most important tools in biological research. The detection of a specific DNA sequence starting from small amounts of DNA requires the design of primers flanking the sequence (Mori et al, 1999). **Random amplified polymorphic DNA** (RAPD) (Welsh and McClelland, 1990; Williams et al, 1990) which uses one or more arbitrarily chosen primers to amplify unknown regions from a given genome.

RAPD analysis can detect the modification of a single base in the genomic DNA, in some conditions. These techniques have become very popular due to their ability to easily and

rapidly generate polymorphic markers using very small amounts of starting DNA, independently of any prior knowledge of the target DNA sequence. These features make random amplification technology a tool useful in many areas of genetic research such as gene mapping, individual and strain identification, population genetics and phylogenetics (Micheli et al, 1997).

Previous studies have shown that changes in band patterns observed in DNA fingerprint analyses reflect DNA alterations from single base changes (point mutations) to complex chromosomal rearrangements (White et al., 1990; Welsh et al., 1991; Atienzar et al., 1999). Similarly, in the present study, DNA damage induced by UV-radiation was reflected by changes in RAPD profiles; variation in band intensity, disappearance of bands, and appearance of new PCR products occurred in the profiles generated by exposed organisms. The variation in band intensities and the disappearance of bands may be attributed to the presence of DNA photoproducts (e.g. pyrimidine dimers, 6-4 photoproducts), which can act to block or reduce (bypass event) the polymerization of DNA in the PCR reaction (Donahue et al., 1994; Nelson et al., 1996).

Method and Materials

Microorganism

Bacillus amyloliquefaciens was obtained from culture collection of Clonegen Biotechnology Private Limited, Noida. The culture was maintained on LB (Luria and Bertani) agar plates and stored at 4°C in refrigerator which was sub-cultured to new agar plates every 15 days.

Strain improvement by UV mutagenesis

Bacillus amyloliquefaciens strain was improved by mutagenesis as described by (Shah et al., 1989). Twenty LB agar plates were spreaded by 50 µl of culture broth of Bacillus *amyloliquefaciens* and exposed to UV light (power of light 15 W , wavelength of 254 nm , distance of 20 cm) for 1 to 20 minutes (Dong et al., 2011; Ramamurthy et al., 1992; Khattab et al., 2012). After irradiation, the treated cultures were protected from light for two hours. Four mutant strains selected **M1 (5 mins), M2 (10 mins), M3 (15 mins), M4 (20 mins)** on the basis of enzyme activity and big zone of starch hydrolysis test.

Genomic DNA Isolation by CTAB method

The CTAB (cetyltrimethylammonium bromide) method was performed as described by (Lipp et al., 1999) with some modifications. The samples (culture broth) were transferred to a 2 mL sterile reaction tube followed by the addition of 1 mL of CTAB extraction buffer (20 g CTAB/L, 1.4 M NaCl, 0.1 M Tris– HCl, 20 mM EDTA) and mixed. After the incubation at 65 ^{0}C for 1 h, with occasional stirring, the suspension was centrifuged (15 min, 18,514g) and 500 µL of the supernatant were extracted with 400 µL of chloroform, stirred and centrifuged (10 min, 12,000g). The upper phase was transferred to a new tube, mixed with double volume of CTAB precipitation

solution (5 g/L, 0.04 M NaCl) and incubated for 1 h at room temperature. After centrifugation (10 min, 12,000g), the supernatant was discarded and the precipitate was dissolved in 350 µL of 1.2 M NaCl and extracted with 350 µL chloroform. The mixture was centrifuged (10 min, 12,000g) and the upper phase was mixed with 0.6 volume parts of isopropanol at 20 ^{0}C. The mixture was centrifuged (10 min, 12,000g), the supernatant was discarded and the pellet was washed with 500 µL of ethanol solution (70% v/v) at -20 ^{0}C.After centrifugation, the supernatant was discarded carefully by pipeting, the pellet was dried and the DNA was dissolved in 100µL of Tris–EDTA buffer (10 mM Tris, 1 mM EDTA) (Mafra et al., 2008; Via and Falkinham, 1995)

Quantification of DNA

The DNA concentration and purity were determined by gel electrophoresis using (0.8 % agarose and TAE buffer and spectrophotometrically by measuring the DNA absorbance and A260/A280-ratios, respectively (Di Pinto et al., 2007; Gupta et al., 2011). One optical density at 260 nm is equal to 50µg/mL DNA (Sambrock et al., 1989). 1mg/ml of Calf thymus DNA in distilled water and prepared different dilution 50-100µg/ml (DNA Standard). Absorbance was taken at 270 nm for DNA Standard. The unknown samples were measured at 260nm and 280nm using UV-VIS Spectrophotometer 119, Systronics.

Amplification by RAPD-PCR

DNA amplification reactions were performed in 25 µl volumes containing 2µl of template DNA, 2.5 µl Taq buffer (100mM Tris (pH 9.0), 500 mM KCl, and 1% Triton X-100 with 15mM MgCl2), 2.0 dNTP µl mix (Bangalore Genei, India), 0.33 µl Taq DNA polymerase (Bangalore Genei, India), 1.0 µl primer and 7.80 µl distilled water. The samples were amplified in a thermal cycler (Eppendorf, Germany) through initial denaturation for 4 mins at 94 °C, 35 cycles of denaturation for 1 min at 94 °C, annealing for 35 ^{0}C and elongation for 90 s at 72 °C followed by final extension for 10min at 72 °C and finally 4 °C for hold (Gupta et al., 2011; Williams et al., 1990 & 1993).

Agarose Gel Electrophoresis

1% w/v agarose was dissolved in 50 ml of 1x TAE buffer (40 mM Tris- acetate, 1mM EDTA) by heating in microwave oven for 1 minute. Keep it for cooling sometime then add 2µl Etbr carefully. Now samples were prepared by mixing 2 µl loading dye, 50 % glycerol, 1mM EDTA and 0.4% bromophenol blue and 0.4% xylene cyanol. Assemble electrophoretic unit (Bio-rad) and add 1x TAE buffer .Always load standard marker in first well and then sample. Run the gel at 85 volt for 50 minute. The gel was visualized and photographed under UV Transilluminator (Moyo et al., 2008; Di Pinto et al., 2007; Thakuria et al., 2008; Gupta et al., 2011).

Data analysis of RAPDs

RAPD fragments were scored as either present (1) or absent (0). The Jaccard's coefficient (Jaccard et al., 1908) was used to calculate the similarity coefficients between each pair of genotypes for all polymorphic loci using the following formula

$$J = \frac{M_{11}}{M_{01} + M_{10} + M_{11}}.$$

Where M_{11} is the number of common bands (1, 1); M_{10} is the number of bands present in the first accession and absent in the second (1, 0); and M_{01} is the number of bands absent in the first accession and present in the second (0, 1). (Raghunathachari et al., 2000).The Jaccard's coefficients between each pair of accessions were used to construct a dendrogram using the unweighted pair group method with arithmatic averages (UPGMA) (Garcia et al., 1999; Kumar et al., 2010).

Result and Discussion

Primer

A single primer with 5'-3' sequence is needed for RAPD reaction, and we used the following single primers for amplification. RAPD primers had been used by (SIGMA-GENOSYS).

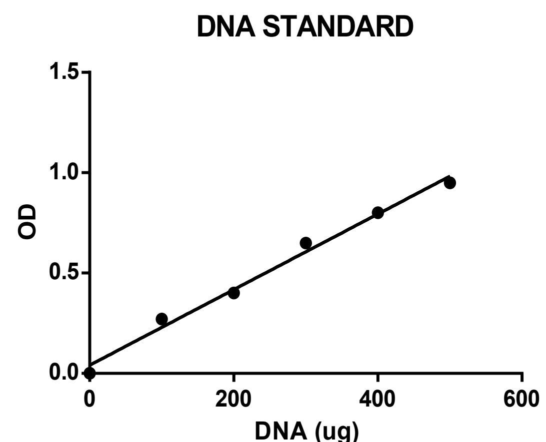
Table 1 Decamer sequences of primers used in RAPD reaction

No.	Primer	Sequence	Tm (^{0}C)	GC%
1	VAA 06	GGTCCCTGAC	30.8	70
2	VAA 16	AGCCAGCGAA	40.5	50
3	VAA 17	GACCGCTTGT	33.7	60
4	VAA 18	AGGTGACCGT	31.6	60
5	VAA 19	CAAACGTCGG	36.6	60
6	VAA 20	GTTGCGATCC	34.2	60

DNA Quantification

Diphenylamine (DPA) Method

The DPA method is carried out by preparing a set of solutions with known DNA concentrations and mixing them with DPA reagent. A standard curve made and the concentration of unknown DNA sample can be derived from the standard curve.



Graph 1 Standard curve of DNA

The concentration of DNA by DPA method represented (Table 2). Wild strain was observed as low quantity of DNA (32.42 µg/ml) than other mutant strains. All four mutant strains had much better quantity of DNA. On the basis of (Table 2) M1 was found as highly concentrated DNA (57.91 µg/ml) in comparison W strain. The DNA concentration of M2, M3 and M4 were observed (39.32, 55.26, 44.63 µg/ml) respectively.

Table 2 Concentration of DNA by Diphenylamine Test (DPA)

Strain	DNA Concentration (µg/ml)
W	32.42
M1	57.91
M2	39.32
M3	55.26
M4	44.63

3.2.2 Ratio of absorbance (A_{260} / A_{280})

A ratio of absorbance (A_{260} / A_{280}) in the range 1.8–2.0 indicates a high level of purity. Wild and all four mutant strains represented as pure DNA because they had ratio from 1.7 to 2.0.

Table 3 DNA Concentration by A_{260} / A_{280} ratio

Strain	Absorbance at 260nm	Absorbance at 280nm	A_{260} / A_{280}
W	1.778	1.001	1.77
M1	2.568	1.310	1.96
M2	2.755	1.502	1.83
M3	2.436	1.230	1.98
M4	2.121	1.200	1.76

Agarose gel electrophoresis

The purity of DNA was determined by gel electrophoresis. All lanes showed bands, that's clearly indicated presence of concentrated amount of DNA. Wild strain, M2, M3, M4 gave sharp bands and M1 showed more dense and sharp in comparison of other strains.

Data analysis

These data were used for the calculation of pair wise genetic distances between trees using the Jaccard coefficient similarity matrices. From all the tested primers in the amplification reactions, the primers VAA 18 gave a good amplification, obtaining a different number of patterns. . The VAA 18 primer yielded a total of 15 bands of which 9

were considered as polymorphic. For observing the similarity degree between the individuals from the two varieties and calculated the matrix similarity based on Jaccard coefficient similarity coefficient.

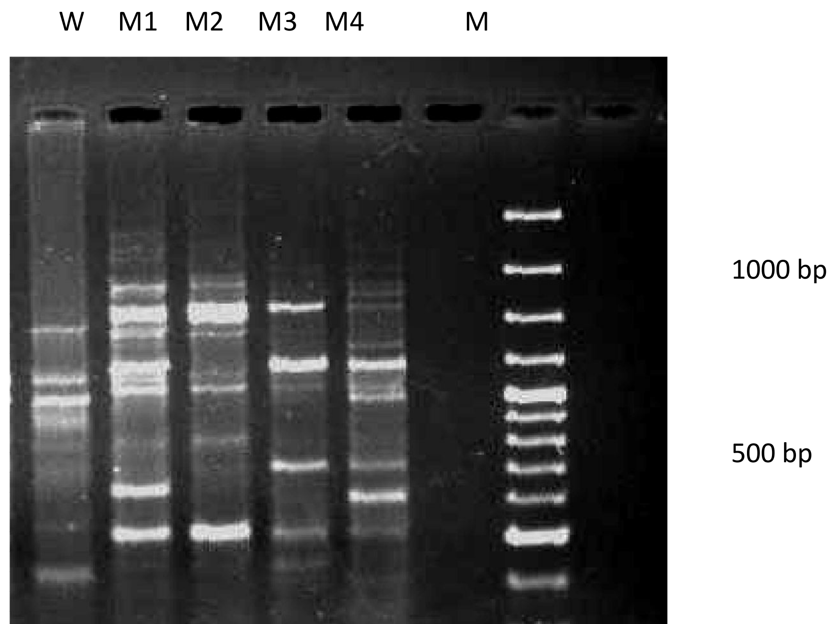


Figure 1 Rapd profile with VAA 18 (primer), the PCR products resolved by electrophoresis on 1% agarose. Designations of strains are indicated on the top: W (wild strain), M1, M2, M3 and M4 (mutant strains) from the left and DNA ladder (1000 bp) shown on the right.

Table 4 Similarity matrix computed with Jaccard coefficient among the five strains

	W	M1	M2	M3	M4
W	1	0.000	0.000	0.000	0.000
M1		1	0.333	0.000	0.143
M2			1	0.000	0.200
M3				1	0.333
M4					1

Table 5 Distance matrix based on Jaccard coefficient

	W	M1	M2	M3	M4
W	0	1.000	1.000	1.000	1.000
M1		0	0.667	1.000	0.857
M2			0	1.000	0.800
M3				0	0.667
M4					0

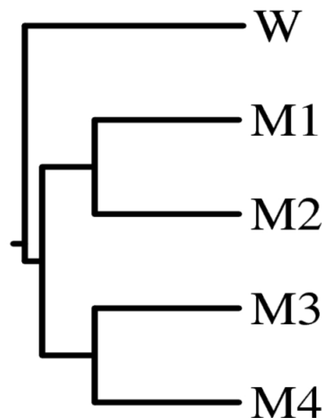


Figure 2 Dendrogram by UPGMA

The method used – UPGMA (Unweighted Pair Group Method with Arithmetic Mean) involves an analysis of the group or the clustering and indicates the relatable connections between the operational taxonomic units by similarity establishing and the difference between all the obtained polymorphisms, with primers which made the amplification. The dendrogram showed two distinct clusters. One cluster formed by Wild strain (W), which exhibited differences from all four mutants. Another cluster included, which divided into two sub- group. First sub-group contained M1 and M2, (Mutant 1 and Mutant 2 had some similarities). Second sub-group had M3 and M4 (Mutant 3 and Mutant 4, they also had similarities.

Conclusion

The results obtained after the dendrogram analysis which indicated the fact that the genetic similarity showed by all four mutant strains but not with wild strain. The group of wild strain was completely distinct from another. Another group was divided into two sub-groups because mutant strain M1 and M2 had been given UV exposure 5 and 10 minutes respectively. That's why they had similarities and found in one group but also showed dissimilarities with M3 and M4. UV exposure on M3 and M4 were 15 and 20 minutes respectively. Thus, it was confirmed on the basis of dendrogram of mutant strains had some genetic changes or mutation by UV mutagenesis.

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