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Isolation, Screening and Molecular Characterization of Polygalacturonase Enzyme from Bacillus Species using RAPD Markers

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***Abstract*— Pectic substances form the major components of the plant cell wall. Polygalacturonase is a hydrolytic enzyme, which acts on polygalacturonic acid (PGA), hydrolyzing α-1,4 glycosidic bonds of pectic acid cleaving the α (1-4) bonds between adjacent galacturonic acid within the homogalacturonic acid backbone of pectin. Pectin modifying enzymes are formed in huge amount by plant related Microbes. Polygalacturonase enzyme has vital role in extraction and softening of fruits, and also plays a major role in agriculture and environmental sectors. The potential of using microorganisms as a biotechnological source in the production of industrially relevant food and textile processing enzymes, has stimulated renewed interest in the exploration of extracellular enzymatic activity. Production and scale up of Bacterial enzymes are easy, low cost and can act on a wider range of pH and environment. The current research was aimed at isolating pectinolytic *Bacillus* sp. from municipal dump sites, screen for polygalacturonase enzyme production and to determine the genetic variability. Six different soil samples were collected and plated on nutrient agar and the isolated colonies were screened for polygalacturonase production on modified MS medium. Of the 20 isolates screened, 8 isolated exhibited clear zones of hydrolysis indicating positive activity for polygalacturonase enzyme. The organisms were identified as *Bacillus* sp. based on colony, cell and biochemical characterization. The genetic variability of these polygalacturonase enzyme producing *Bacillus* sp. were analyzed using PCR based RAPD technique using four primers RBA1, RBA2, RBA3, RBA4. Three different groups of RAPD profiles were determined within the eight species based on the distance calculated by Euclidean Similarity matrix. The First clade has 4 organisms with ORG13 and ORG18 closely related followed by ORG1 and ORG15 and in the second clade ORG4 and ORG12 are closely related and according to the third clade ORG16 and ORG17 were found to have close evolutionary relationship. The genetic diversity is evident based on the percentage similarity between the isolates.**

***Keywords*— Polygalacturonase, Pectinase, Bacillus, RAPD, Molecular Markers**

I. INTRODUCTION

Enzymes play crucial roles in almost all the bio-processing and biotechnological industries as a biocatalyst. Nowadays, use of bioenzymes is creasing as they show promising benefits and unique characteristics as compared to the synthetic one. Its is experimentally proven that bacteria are the new, robust and cost-effective source of the bioproduction of enzymes (Bibi N. *et al*., 2018).

Among various enzymes that are produced biologically from bacterial or prokaryotic system, pectinases especially polygalacturonases are industrially important enzymes (Bibi N. *et al*., 2018). Polygalacturonases which are the type of depolymerizing enzymes from pectinase family are present in two sub-forms such as endo-polygalacturonases and exo-polygalacturoases (Aarti C. *et al*., 2015). These enzymes functions by hydrolyzing the internal and external α (1,4)-glycosidic linkage between two galacturonic acid residues (Aarti C. *et al*., 2015 & Zeni J. *et al*., 2011). The galacturonic acid is a major component of polygalacturonan which is an important structural component of pectin network. Plant cell walls comprise of pectin (Zeni J. *et al*., 2011). Pectins are high molecular mass versatile polysaccharides and responsible for the structural integrity and cohesion of plant tissues (Voragen *et al*., 2009). The endogenous polygalacturonases of plant help in softening process during ripening of fruits. Similarly, bacterial polygalacturonases function in weakening and rotting of plant materials (Greg Tucker. *et al*., 2017).

The enzyme polygalacturonase which possess very efficient characteristics of degrading pectin are widely used in many biotechnological and industrial purposes (Patidar MK. *et al*., 2018). The textile industries which perform degumming and retting of fibers, the paper and pulp industries use bacterial isolated polygalacturonases to improve the quality of the yield. Our target enzyme is also used vastly in extraction of juice and oil, fermentation of wine, coffee and tea. This enzyme plays important role in enzymatic treatment of waste water (Lü F. *et al*., 2016). The enzyme polygalacturonase is considered as the most

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effective and efficient biocatalyst for hydrolysis of pectin (Rahman MS. e*t al*., 2019). Many industries and biotechnology sectors have started concentrating on the application of this enzyme in various purposes as the enzyme works with a favorable efficiency and also minimizes fiber damage (Rahman MS. *et al*., 2019). The demand of commercial polygalacturonases with novel biochemical and physicochemical characteristics is increasing day by day for being relevant for several biotechnological industries such as bio-pharmaceutical, textile, brewery industries.

Further exploration of new source of polygalacturonases is increasing. Hence, use of bacterial and fungus source for the production of this enzyme comes in picture (Jayani. *et al*., 2010).

A bacterial strain *Bacillus sp.* shows promising feature for the production of commercial polygalacturonases (Swain MR. *et al*., 2009 & Jayani. *et al*., 2010). Bacterial polygalacturonase from *Bacillus sp*. contributes to approximately 50-60% of the global market for enzymes (A.R.E.O. Xevier *et al*., 2017). *Bacillus sp* which are gram positive, rod shaped, aerobic or facultatively anaerobic, ubiquitous microorganisms can grow on natural media and helps in producing high yield of enzymes in a cost-effective way (Gaurav Pant. *et al*., 2015). Studies have suggested that many *Bacillus sp.* such as *Bacillus lichenformis* can produce polygalacturonases efficiently (Preeti G. *et al*., 2013).

To study the ecological distribution and molecular characterization of different Bacillus strains the random amplified polymorphic DNA(RAPD) techniques is widely used (Boroujeni *et al*., 2012). This technique which includes RAPD marker is very useful of determine intra-species variations. The RAPD markers are 8-15 nucleotide long DNA fragments which are generated by a polymerase chain reaction (PCR) amplification of random fragment of genomic DNA with single primer (Mkada driss I *et al*., 2014). The RAPD technique is considered as the tool for designing molecular markers which are specific to particular taxon. The genetic characterization of any species is achieved by the sequencing of specific RAPD markers (Mkada driss I *et al*., 2014 & Verma KS. et al., 2017).

In this particular study, we aimed to detect the molecular or genetic characteristics of soil dwelling *Bacillus* sp. which are capable of polygalacturonase production by RAPD molecular markers.

II. MATERIALS AND METHODS

2.1 Sample collection

The soil samples were collected from different community dump sites where rotten fruits and vegetables were degraded in air tight sterile zip-lock bags for the isolation of polygalacturonase producing organisms, transported to the laboratory and stored under refrigerator conditions until further use.

2.2 Isolation and identification of *Bacillus sp.*

1.0gm of collected samples were mixed in 100 ml of 1N saline in different conical flasks. Serial dilution in the range of 10^{-1} to 10^{-6} was done and 0.1 ml of different diluted samples were spread on Nutrient agar plates. The plates were incubated for 24h at 37 ˚C. The colonies were identified based on morphological and biochemical characters according to Bergey's manual of determinative bacteriology, which were identified as *Bacillus* sp. The obtained colonies were sub cultured using nutrient agar slants for further analysis.

2.3 Screening of isolates for polygalacturonase production

The pure culture of isolates was spot inoculated on 0.2% pectin supplemented Minimal Salts agar media and incubated for 24h at 37 ˚C. The isolates were observed for the formation of zone of clearance which indicates the production of polygalacturonases.

2.4 Genomic DNA isolation

The genomic DNA isolation from the isolated organisms was done by phenol chloroform method. Centrifugation of fresh bacterial cell suspension (2ml) was done at 4°C, 15000g for 10 minutes. The supernatant was discarded and the pellet was resuspended in a lysis buffer containing 0.05mM Tris-HCl (pH-8.0), 0.05mM EDTA, 0.1mM NaCl, 2% SDS, 0.2% PVP and 1% mercaptoethanol to lyse the cells. Then a equal volume of phenol (pH-8.0), chloroform, isoamyl alcohol (25:24:1) mixture was added to eliminate proteins. Then the mixture was centrifuged at 15000g for 15minute at 4°C to separate aqueous layer. The aqueous layer was transferred into a fresh vial and equal volume of chloroform: isoamyl alcohol (24:1) was added to it followed by invert mixing and centrifuge at 15000g, 4°C for 10 minutes. The upper layer obtained post centrifugation was transferred to a fresh vial, to which an equal volume of Chilled ethanol (99.9%) was added and incubated overnight at -20°C. The DNA was pelleted down by centrifugation at 15000g for 10 minutes at 4°C. The DNA pellet washed with 70% ethanol for eliminate impurities such as salts. Then the pellet was allowed to air dry to evaporate ethanol and the pellet was dissolved completely in adequate amount of autoclaved distilled water. Quantification and purity of isolated DNA was done by using a nanodrop spectrophotometer at wavelength 260/280.

2.5 Molecular characterization using RAPD-PCR analysis

The bacterial isolates were screened by RAPD-PCR analysis using 4 random oligonucleotide primers for determination of their polymorphism. The PCR

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amplification reaction mixture was prepared in a total volume of 25µl including 1µl of DNA, 2.5µl of 10xTaq polymerase buffer with $MgCl_2$, 1.5µl 2.5mM dNTPs, 2µl Taq polymerase, 1µl of 10pmol RAPD primers. The PCR reaction was performed in a thermal cycler for 4 minutes of denaturation at 95°C followed by 45 cycle for 1 minute at 94°C, 1minute at 37°C and 1 minute for 72°C. The final extension was done for 10 minutes at 72°C.

2.6 Analysis of PCR amplified product

The PCR products were separated in 2% agarose gel. The DNA bands were visualized in UV transilluminator and compared with 100bp DNA ladder.

2.7 Data interpretation and determination of evolutionary relationship

The distance matrix was computed and saved using the software PyElph from the data generated. The binary score value was used by PAST 325 software to construct the final dendrogram showing diversity among all species of the isolated organism with all primers.

III. RESULT

3.1 Isolation and identification of target organisms from soil samples

Six different soil samples were collected from the community dump site of Ullal, Bangalore and covered in sterile zip-lock bag. Bacteria isolated from the soil samples were grown on nutrient agar media. The bacteria showing some physiological characteristics such as white or creamish in color, dry, flat or elevated and irregular colonies were found to be Gram's positive bacilli on staining and were further screened for biochemical assays. 20 *Bacillus sp.* were identified and screened for polygalacturonase assay.

3.2 Screening of isolated organisms for polygalacturonase production

Out of 20 *Bacillus sp.* 8 such organisms (1,4,12, 13, 15, 16,17,18) showed maximum zone of clearance on the 0.2% pectin supplemented MS-agar media were identified for production of extracellular polygalacturonase production (Fig. 1). Those isolates were named and subjected to further RAPD analysis.

3.3 Morphological and biochemical characterization

Those selected strains were morphologically and biochemically characterized according to Bergey's manual of determinative bacteriology. The strains were identified as *Bacillus sp*.

3.4 Isolation and estimation of genomic DNA

The genomic DNA were isolated from the selected strains and separated on agarose gel. The DNA bands were visualized under UV transilluminator. Clear DNA bands were visible without RNA or protein contamination and the yields of genomic DNA were obtained (Fig.-2). DNA isolated from the organisms were examined at 260/280 wave length ratio which showed the presence of pure DNA (ratio of absorbance ~ 1.8).

3.1 PCR analysis of RAPD (random amplification of polymorphic DNA)

The RAPD were performed using primer RBA1, RBA2, RBA3, RBA4. The PCR (polymerase chain reaction) products were separated on a 2% agarose gel and compared with 100bp DNA ladder. The banding patterns were observed based on the presence and absence of clear visible and reproducible bands (Fig. 3). The bands were analyzed on the basis of polymorphic and monomorphic DNA principle.

3.5 Construction of dendrogram to determine evolutionary relationship

The tree was computed based on the information extracted from the gel image and the genetic distances were displayed on the branches. The distance matrix corresponding to the gel image was computed and saved using the software PyElph (Ana and Cristian, 2012). Further, the binary score value was used by PAST 325 software to construct the final dendrogram showing diversity among all species with all primers (Øyvind, 2019). The program used was cluster analysis joining (tree clustering) with raw input data of each population separately. The main parameter, which guided the joining process linkage rule, is unweighted pair group average (UPGMA) and the genetic distance was estimated from raw data. Genetic distance denotes the genetic divergence between species or between populations within a species. It is measured by a variety of factors. Smaller genetic distances indicate relativeness whereas large genetic distances indicate a more detached genetic relationship (Table - 1).

8 organisms were divided into 3 clades depending on the evolutionary relationship based on the distance calculated by Euclidean Similarity matrix. First clade has 4 organisms where in ORG13 and ORG18 are closely related followed by ORG1 and ORG15. In the second clade, ORG4 and ORG12 are closely related and the third clade consists of ORG16 and ORG17 showed close evolutionary relationship as predicted by past325 software (Fig. 4).

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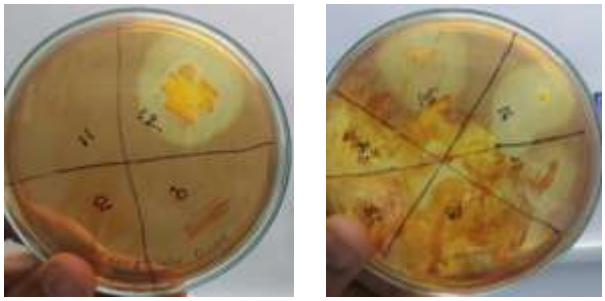

(a) (b)

Figure 1. (a) strain number: 9, 10, 11, 12. Out of which only strain 12 showing zone of clearance, (b) Strain number: 13, 15, 16, 17, 18 showing the zone of clearance.

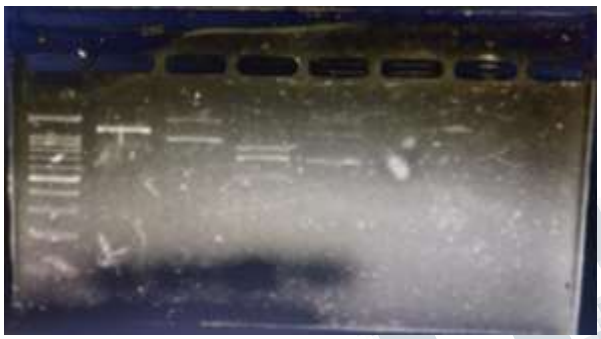

Figure 2. Agarose gel image of isolated genomic DNA

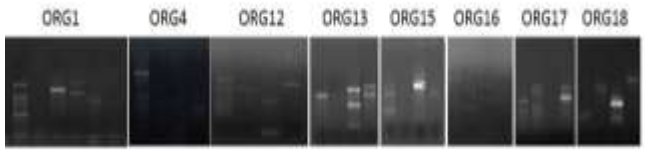

Figure 3. Gel image showing PCR profiles of Organisms using primer 50, 51, 52 , 53 and compared with 100bp ladder on 2% agarose gel.

Table 1. Euclidean Similarity and distance indices obtained in PAST 325 using which Dendrogram was constructed.

	ORG 1	ORG 4	ORG 12	ORG 13	ORG 15	ORG 16	ORG 17	ORG 18
ORG1	0	3.87	3.60	3.74	3.31	3.46	3.87	3.16
ORG4	3.87	0	3.16	3.60	3.16	3.31	3.74	3.60
ORG12	3.60	3.16	0	3.60	3.16	3.31	3.46	3.31
ORG13	3.74	3.60	3.60	0	3.31	4	4.12	3.16
ORG15	3.31	3.16	3.16	3.31	0	3.31	3.46	3.31
ORG16	3.46	3.31	3.31	4	3.31	0	3.60	3.16
ORG17	3.87	3.74	3.46	4.12	3.46	3.60	0	3.87
ORG18	3.16	3.60	3.31	3.16	3.31	3.16	3.87	0

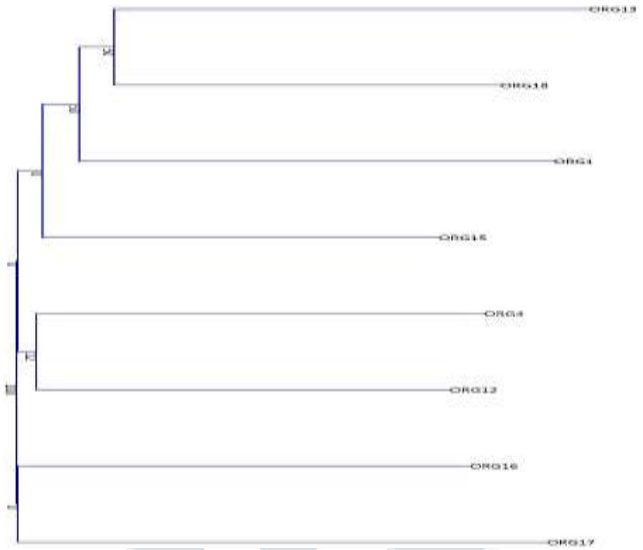

Figure 4. Dendrogram showing clustering of 8 organisms constructed using UPGMA based on Euclidean Similarity matrix.

IV. DISCUSSION

Our targeted organism *Bacillus sp*. are the most abundant microbes in the nature. Several products including enzymes, antibiotics, which are industrially important are produced naturally by *Bacillus sp.* (Perez KJ. *et al*., 2017). For this study, the bacterial sample was collected from soil of community dumping site in Bangalore and characterized according to the Bergey's manual of determinative bacteriology (Williams & Wilkins, 1986). The isolated organisms were qualitatively screened using 0.2% pectin supplemented MS-agar media to determine the production of polygalacturonase. Out of 20 selected bacilli strains only 8 strains were found to produce polygalacturonase. RAPD-PCR analysis was done using four different primers to determine evolutionary relationships between different strains of *Bacillus sp*. The RAPD-PCR technique which has been considered as gold standard tool can generate taxon specific markers (Kwon GH. *et al*., 2009). Based on the RAPD data the dendrogram for polygalacturonase producing *Bacillus sp.* Strains was constructed.

V. CONCLUSION

In this study RAPD-PCR analysis showed a positive phylogenetic relationship between the selected Bacilli strains which are capable of producing polygalacturonase. Using RBA1, RBA2, RBA3, RBA4 primers the genetic variability among the bacterial strains were also determined. RAPD technique can be more useful tool to detect evolutionary relationship especially molecular characteristics between different species of any organism.

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