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Isolation of High Quality DNA from Pulverized Rice Grain Samples by Tri-Phase Method

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Abstract: ***Genetic studies in plants require the isolation of high quality DNA from large number of plant sources. Commercial DNA extraction kits are convenient and usually safe, but their availability to certain developing countries and high cost of available kits can be limiting factors, especially when handling a large number of samples and considering experiments with limited financial resources. There has been a need for reliable, inexpensive and safe method to isolate DNA from milled/pulverized rice samples. Hence, a simple, economical and reliable method is presented to isolate high quality DNA from milled rice samples. DNA isolation is conducted in three phases; cell lysis phase, DNA precipitation phase and de-proteinizing phase. Initially cell lysis is done by extraction buffer, later K+ ions in Potassium acetate helps in replacing Na+ ions. Hence, dodecyl sulphate coated starch and protein complexes become precipitated. Further DNA purification is done by de-proteinizing treatment with phenol: chloroform: ethanol, mixture. DNA obtained by this method can be subjected to enzymatic reactions, such as digestion with restriction enzymes, ligation and PCR amplification.***

Keywords: SDS (sodium dodecyl sulphate), genomic DNA, Molecular weight (MW), Polymerase Chain Reaction (PCR)

1. Introduction

An indispensable task in any molecular biology laboratory is the isolation of genomic DNA (gDNA). The Cetyl Trimethyl Ammonium Bromide (CTAB) method has been common in isolating DNA from most of the leaf samples. But, none of the methods have been so popular in isolating DNA from grains/seeds. This is mainly due to the high starch components in the samples. In the case of rice (*Oryza sativa*) there are many rapid and efficient protocols available for DNA extraction from leaf samples but from milled rice samples, DNA extraction has been possible only with the commercially available kits such as Nucleon PhytoPure DNA Extraction kit [1], [2]. DNA isolation from pulverized rice samples has been difficult because of minute amount of DNA left in milled rice, as bran layer and embryo are stripped away during milling. The occurrence of higher levels of polysaccharides (>90% starch) and DNA shearing and/or degradation may be happened during the processes of desiccation, storage and milling of mature rice grains.

The DNA could be isolated from grains of plant species, using a single protocol, a method relevant to an open laboratory environment with minimal equipment requirements and waste output. Commercial DNA extraction kits that do not use hazardous reagents have these idyllic properties; however, their expediency and safety may be cost exorbitant when considering experiments with limited fiscal resources. Furthermore, in some instances, commercial kits have produced low DNA yields and capricious quality [3], [4], [5], [6], [7].

Contaminants such as tannins, polysaccharides, and pigments can inhibit the annealing of DNA or the enzymatic activity of restriction endonucleases [8], [9]. This is a problem for many technical applications including PCR and Southern analysis. Quite a few non-toxic extraction methods use high salt concentration buffers, proteinase K treatments, or DEAE-cellulose to obtain a higher purity of DNA [5], [10], [11]. DNA isolated by using methods is suitable for PCR applications but auxiliary purification is required for techniques that involve the enzymatic digestion of DNA. These methods are also less effectual in plants rich in polysaccharide and polyphenolic compounds.

Thus, objective of this study was to develop a reliable, inexpensive method to isolate DNA from starch rich compounds, without using toxic chemicals. The resulting DNA could be used for further PCR analysis and restriction endonuclease digestion.

2. Materials and Methods

Sample grains of IR, Basmati and *pokkali* were collected from private farms. The grains were milled and stored in deep freezer (-200 C), till the date of experiment.

2.1 Cell Lysis Phase

0.5 g of pre-chilled pulverized rice samples were ground to fine powder in liquid nitrogen and transferred to 2ml tube. Each Sample received 800 µl of pre-warmed (at 650 C) extraction buffer (400 mM Tris HCl pH 7.8, 60 mM EDTA pH 7.8, 500 mM NaCl, 1.25% SDS, and 0.38% Sodium bisulfate) and 50µg RNase A. Then 250 µl of 5M potassium acetate was added, and mixed well. Each tube was incubated at 600 C for 30 mins gently inverting occasionally; final pH of the sample was maintained at 7.8-8.0. Add 800 µl of chloroform, and samples were incubated on ice for 20 mins. Samples were centrifuged at 12000 rpm for phase separation for 10 mins at room temperature.

2.2 DNA precipitation Phase

The supernatant was transferred to a clean tube, and equal volume of isopropanol was added. Sample was kept at 40 ^{0}C for three hours to ensure complete precipitation. The precipitated DNA was centrifuged at 12000 rpm for 10 mins

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at room temperature and the pellets were washed with 0.5 ml of 70% ethanol and left for air drying. Air-dried DNA pellets were then re-suspended in minimum amount of TE [10 mM Tris HCl (pH 8.0), 1mM EDTA (pH 8.0)]. DNA samples were centrifuged at 12,000 rpm for 10 min to get rid of starch and other insoluble components.

2.3 De-proteinizing Phase

De-proteinizing reaction was the next process; hence the samples were transferred to new 2 ml Eppendorf tubes and a mixture of 1 ml phenol: chloroform: ethanol = 25: 24: 1 was added. DNA was again precipitated using Isopropanol and kept in ice for 1 hr. Precipitated DNA was centrifuged at 10000 rpm for 10 minutes at room temperature and pellets were air/vacuum dried and dissolved in 50-200 µl of TE buffer. This DNA can be stored in -200 C and used for future purpose. All the processes were carried out in TE buffer, slight change in ionic content could result in the failure of the experiment.

The isolated DNA from three rice samples was fractionated on 1.0% agarose gel. Lane-1 consisted of Hind-III digests of λ DNA as molecular marker. Lane 2, 3 and 4 were loaded with DNA samples of Basmati, IR and *Pokkali*. The samples were allowed to run, until clear bands were achieved **(Figure-1)**.

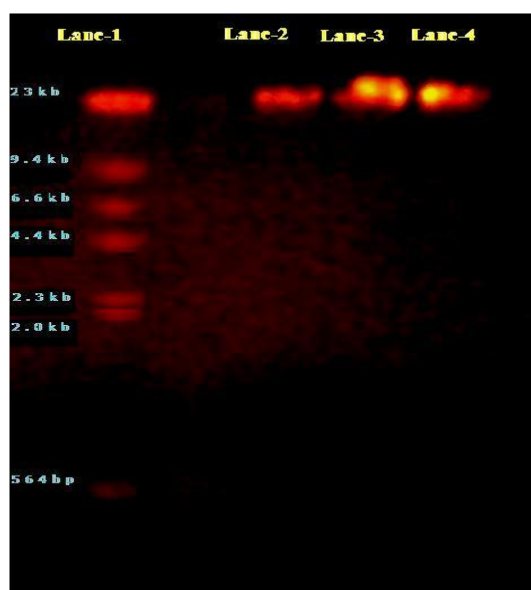

Figure 1: Isolated raw DNA from rice grains were fractioned on 1.0% Agarose and stained with Ethidium Bromide. Lane-1, Hind-III digests of λ DNA as a molecular weight marker. The largest band is 23kb in length. Lane 2, 3 and 4 consist of DNA's isolated from Basmati, IR and *Pokkali.*

The DNA's obtained from Basmati, IR and *Pokkali* have been used as the templates in PCR amplification of RM231 rice SSR marker, as our work focuses on genetic diversity studies of *O. sativa* **(Figure-2).**

Figure-2: PCR amplified DNA fragments using RM231 rice SSR marker. 17 accessions of *O.sativa* were used in the study (Lane-1-17).

3. Results

The physical characteristics of the final DNA pellet were white with no visible discoloration. The electrophoretic pattern of the isolated DNA showed a clear single band, signifying that fragmentation of DNA into small sizes seldom occurred during the process of isolation **(Figure-1).** Lane-1 consisted of Hind-III digests of λ DNA as molecular marker. Lane-2, 3 and 4 consisted of DNA samples of Basmati, IR and *Pokkali* respectively. DNA's obtained by this method clearly serves as templates in PCR amplification of RM231 rice SSR marker **(Figure-2).** Band intensities of different bands have been analyzed by gel analyzer, 2010 **(Figure-3).**

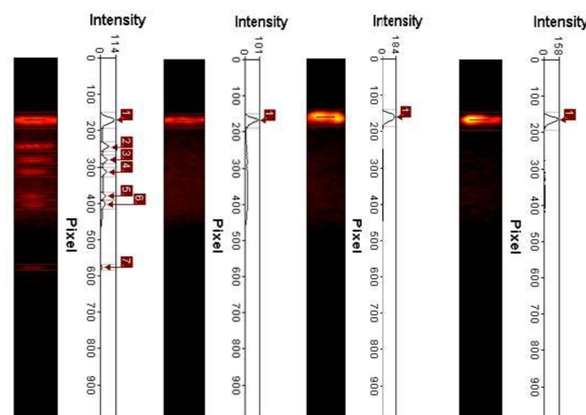

Figure 3: Comparison of band intensity in different Lanes.

Density of the samples was estimated using Gel Documentation Software, here the density of the bands of the marker DNA was assumed to be 100%. Density of the samples in lanes-2, 3, and 4 were estimated to be 48.90, 33.67 and 33.36 respectively **(Table-1).**

Table 1: Densitometry Tracing Table comparing the three sample lanes with standard lane (bands in lane-1 are placed constant at 100%).

Band	Standard Lane-1(%)	Sample Lane -2 (%)	Sample Lane –3 (%)	Sample Lane-4 (%)
1	100.00	48.90	33.67	33.36
2	100.00	-	-	-
3	100.00	-	-	-
4	100.00		-	-
5	100.00	-	-	-
6	100.00	-	-	-
7	100.00	-	-	-

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The volume of the DNA isolated was analyzed using Gel Analyzer 2010, software **(Figure-4).** Comparison of Raw volume and calibrated volume was plotted. DNA sample of IR variety was estimated about 4000. The molecular weight of the DNA bands estimated using **Gel Analyzer Software, v.2010** were; IR showed about 40kb, whereas basmati and *pokkali* bands were around 20kb and 22kb respectively **(Figure-5).**

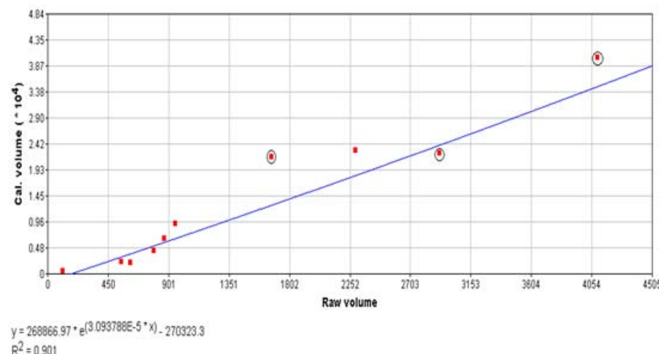

Figure 4: Quantitative analysis Graph, Calibrated volume on x-axis and raw volume on the y- axis. Dots inside the ellipse represent calibrated volume of Basmati, IR and *Pokkali.*

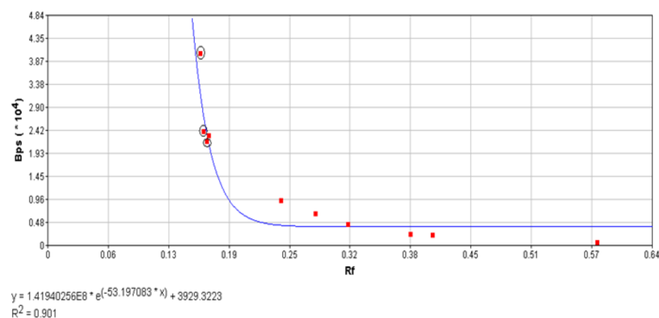

Figure 5: DNA base pair size determination. Dots in the circle represent MW (Molecular Weight) of Basmati, IR and *Pokkali.*

Analysis information of complete DNA samples have been also formulated using **Gel Analyzer, v.2010 (Table-2).**

Table 2: Analysis information of the DNA samples fractioned on 1.0% Agarose

Lane #	*Band #*	*Rf*	*Raw Volume*	*Cal. Volume ($x\ 10^4$)*	*Mol. Weight ($x10^4$)*
1	1	0.170	2292	23000	23000
1	2	0.240	948	9400	9400
1	3	0.282	720	6600	6600
1	4	0.316	602	4400	4400
1	5	0.382	490	2300	2300
1	6	0.405	440	2200	2200
1	7	0.578	101	564	564
2	1	0.168	1669	21870	21760
3	1	0.161	4095	39690	40950
4	1	0.164	2918	22409	22009

4. Discussion

The isolation of high quality DNA is an important step in the field of plant molecular biology. A reliable method to isolate DNA from pulverized rice samples is unavailable; hence the current study focus on a dependable approach to isolate high quality DNA from milled/pulverized rice grains. In the process, cell lysis phase is initially done, later DNA precipitation phase and finally de-proteinizing phase. In the SDS lysis phase, starch and proteins become trapped in complexes that are coated with dodecyl sulphate. Unlike other isolation process here sodium ions are replaced by potassium ions, hence dodecyl sulphate coated starch and protein complexes become precipitated [12], [13]. Proteins and other cellular contaminants are usually removed by phenol: chloroform: ethanol mixture. DNA obtained by this method also serves as a good template for PCR amplifications. This method is safe, inexpensive and reliable when compared to commercial kit (QIAGEN DNEASY PLANT MINI KIT - $760 for 250 samples).

In summary, here we describe a safe, simple, cost efficient and reliable method that provides high quality, protein-free DNA from pulverized/milled rice grains. This method can be applied in other starch/polysaccharide rich compounds.

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