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Quality DNA Isolation Using Different Methods of Sugarcane *(Saccharum officinarum L.)*

M. A. Hossain, M. M. Shaik[1], R. M. S. Shahnawaz[1], N. Islam, M. R. Islam[1] and M. A. S. Miah
Bangladesh Sugarcane Research Institute,
Ishurdi, Pabna, Bangladesh.

ABSTRACT

Three methods were used for DNA isolation from fresh leaf tissues (laminar tissue and meristem cylinder) collected from field grown 8 month old plants. The samples were grind without liquid nitrogen. The quality and quantity of DNA were determined by visual estimation of electrophoreses agarose gel and by spectrophotometer. Method I included use of Ultra-Turax for sample grinding, refrigerated centrifuge for separation of different components of samples and large amount chemicals were used in different steps compared to methods II and III. In both Method II and Method III grinding was done using mortar pestle in polypropylene bag at room temperature. Quality and quantity of DNA were determined by electrophoreses gel observation staining with Ethidium bromide, and spectrophotometer. Different DNA amounts were recorded in different isolation methods. The highest amount of DNA was obtained from the Method III (4100-4600 ngml-1) followed by Method II (1400-1950 ngml-1) and Method-I (1250-1650 ngml-1). Quality DNA isolation was possible without use of liquid nitrogen, tissue homoginizer and using a simple micro-centrifuge.

Key words: Sugarcane, quality DNA, DNA isolation,

INTRODUCTION

Sugarcane is one of the leading sugar crops in the world, producing 70% of the world's sugar. It is the major cash-cum-industrial crop in tropical and subtropical regions of the world and important export product in many developing countries (Heinz *et al.* 1977). Sugarcane is the most important cash crop, especially north and southern part of Bangladesh. Any step that can add to the understanding of sugarcane genome organization and function will assist in increasing sugar production worldwide. Like many other plant species, sugarcane tissues contain high levels of polysaccharides and polyphenolic compounds, which present a major contamination problem in the purification of plant DNA. When cells are disrupted, these cytoplasmic compounds can come into contact with nuclei and other organelles (Loomis, 1974). In their oxidized forms, polyphenols covalently bind to DNA giving it a brown colour and making it useless for most research applications (Katterman and Shattuck, 1983; Guillemaut and Maréchal, 1992). One method commonly used to avoid problems with polyphenols involves freezing the tissue during or prior to homogenization (Katterman and Shattuck, 1983; Leutwiler et al., 1984). The presence of these compounds render studies difficult due to long and tedious extraction procedures and often does not result in good standards in terms of yield and quality. The need for a rapid and efficient procedure for plants having high polysaccharides and polyphenols is necessary when hundreds of samples need to be analyzed in short time, such as in genome mapping and marker assisted selection (MAS) programmes. High purity DNA is required for PCR and restriction-based techniques such as random amplified polymorphic DNA (RAPD), for microsatellite, RFLP and amplified fragment length polymorphism (AFLP), for genome mapping and DNA fingerprinting. Several protocols have been described for plants containing high amounts of polyphenols and polysaccharides including extraction of DNA from isolated nuclei (Hamilton et al., 1972) and purification by cesium chloride following the classic plant DNA protocol (Murray and Thompson, 1980) using liquid nitrogen. Although these methods yield DNA of high purity, they are tedious and require expensive reagents and equipment such as high-speed or ultracentrifuges. Also, liquid nitrogen may be difficult to obtain in certain regions of the world where most species of Saccharum are found. A rapid DNA extraction method for sugarcane and its relatives (Honeycutt *et al.* 1992) and recently, a modification of a CTAB DNA extraction protocol for plants containing high polysaccharides and/or polyphenol components (Porebski *et al.* 1997; Peterson *et al.* 1997),

[1]Department of Biotechnology and Genetic Engineering, Islamic University, Kushtia, Bangladesh.

have been published. These methods give a low yield of DNA and procedures are relatively long, thereby limiting number of samples processed . In addition, expensive reagents and tedious processes were used in these protocols, such as RNase, spermidine, proteinase K and filtration of ground tissue through cheese-cloth and/or mira-cloth. An improved and rapid protocol for isolation of polysaccharides and polyphenol free sugarcane DNA which also includes expensive equipments such as Ultra-Turax and refrigerated centrifuge (Aljanabi *et al.* 1999). Considering the above facts this investigation was undertaken to isolate polysaccharides rapidly and polyphenol-free pure genomic DNA from sugarcane using low cost equipments as well as avoiding liquid nitrogen.

MATERIALS AND METHODS

In this experiment two types of samples such as meristem cylinder and Top Visible Dulap (TVD) leaves of sugarcane were used. Top of the field grown 8 month old sugarcane plants was cut and placed vertically in a bucked with water to preserve alive and fresh. In laboratory, for meristem cylinder outer leaf sheaths were removed leaving inner spindle. Then the spindle base was cut away with sterile scissors and required amount was taken using a fine balance. TVD leaf was taken and mid rib was discarded. Then middle portion of leaf blade was cut into pieces of about 1cm x 1cm size. Then required amount was weighed with a fine balance. For total genomic DNA isolation three different protocols were used (Table 1).

Solutions

- ☐ Homogenization buffer: 200 mM Tris-HCl, 50 mM EDTA, 2.2 M NaCl, 2% CTAB, 0.06% sodium sulfite1, pH8.0
- ☐ Phenol:chloroform:isoamyl alcohol (25:24:1)
- ☐ 6 M NaCl
- ☐ 10% polyvinylpyrrolidone (PVP)
- ☐ 5% N-lauroyl-sarcosine
- ☐ 20% CTAB

RESULTS AND DISCUSSION

Isolation of DNA

Three methods were used for isolation of DNA. The methods were considered based on equipment and chemical without loss of quality and quantity of DNA for lateral use. Fresh leaf tissue and meristem cylinder were used for DNA extraction. Centrifuge, Ultra-Turax, use of liquid nitrogen during grinding and amount of chemicals were important considerations for DNA isolation in different methods. Method I included use of Ultra-Turax for sample grinding, refrigerated centrifuge separation of different components of samples and large amount chemicals were used in different steps compared to methods II and III. This method was adopted with modification of method used by Aljanabi et al. 1999. Method II was the modification and combination of methods described by Aljanabi et al. 1999 and method used by Biogenetics Laboratory of IPB, UPLB, Philippines, 2003. In this method fresh mature leaves were used for DNA extraction. Grinding was performed using mortar and pestle in polypropylene bag at room temperature. Ultra-Turax an expensive equipment was bypassed in this method. After grinding, filtration was done to recover extractant using sterilized duster cloth an easy available and low cost material. Separation of debris and other component of tissues were done using refrigerated centrifuge at 40C of temperature. After centrifugation the aqueous phase was recovered and distributed in different 1.5 ml eppendrof tubes to reduce use of chemicals. This method is comparatively easier than Method I. Method III was the modification of method I, II and combination of two methods. This is the mini-prep method of DNA isolation using little amount of chemicals and without use of Ultra-Turax during grinding, refrigerated centrifuge during separation. In this method samples was grind in sterilized polypropylene bag using mortar and pestle. For separation of others component of samples were done centrifugation the samples using micro-centrifuge of fixed speed (10,000 rpm) at room temperature. This method is the easy and cheaper compared to methods I and II.

Quality and quantity of DNA recovered was pure and significantly different among the methods used. The highest quantity of DNA was obtained from the Method III which was found highly significantly different over the Methods I and II (Table 2). Therefore, the Method-III may be used for DNA isolation of sugarcane since this method is comparatively easy, chemical cost can be saved and cheaper & locally available equipment are need.

Quality of DNA

To check the DNA quality, genomic DNA isolated from Method III was run on a 1% agarose gel for each sample and observed. There was neither RNA or polyphenol contamination nor any sign of degraded DNA in all samples. No RNase treatment was performed in any DNA sample. The detrimental influence of polyphenols and their oxidation products can be counteracted by different strategies. Inclusion of polyphenol absorbants such as bovine serum albumin (BSA) and soluble PVP in the isolation buffer, usually at the concentrations of 1-2% (Bult *et al.* 1992; Couch and Fritz , 1990). The purity of isolated DNA was also determined by spectrophotometric quantification. The DNA purity was measured by dividing absorbance at 260 nm by absorbance at 280 nm. The ratio of absorbance reading at 260 nm (A260) to absorbance reading at 280 nm (A280) i.e. A260/A280 ratio is used for the determination of DNA purity. The A260/A280 higher than 2.0 generally indicates RNA contamination. For A260/A280 ratios lower than 1.8 normally indicates protein contamination during extraction process. However, Aljanabi and co-workers reported range of A260/A280 ratio from 1.76-1.96 for good quality DNA of sugarcane in 1999 (Aljaboni et al.1999). The A260/A280 ratio in Method I was averaged in the range of 1.555-2.272. In the Method-II, A260/A280 ratio was averaged in the range of 0.966-1.444. In the Method III, the A260/A280 ratio in Method III was averaged in the range of 1.518-2.044. In the Method III, out of six samples three yielded good quality DNA. Good quality DNA was recovered from the Method I and III. However, no good quality DNA was recovered from the Method II.

Quantification of DNA

To determine amount of isolated DNA, visual estimation and spectrometric method were used. Visually DNA was estimated by observing DNA in the electrophoreses agarose gel comparing with known marker DNA. In spectrophotometric method amount of DNA was determined by taking absorbance reading at 260 nm and putting this value using formula described below.

$$\text{DNA concentration} = A260 \times \text{Dilution factor} \times \text{Conversion factor}$$

$$= A260 \times \frac{\text{Volume of distilled water } (\mu l)}{\text{Amount of the DNA sample } (\mu l)} \times 50$$

$$= (\mu g ml^{-1})$$

$$= (ng\mu l^{-1}) \quad [\mu g ml^{-1} = ng\mu l^{-1}]$$

Here,

A260 = Spectrophotometric Absorbance reading at 260 nm of DNA sample.

Dilution factor = The ratio of volume of distilled water (ml) to amount of DNA sample (ml).

Conversion factor 50 = The 50mgl-1 of DNA contained in a solution which gives the Spectrophotometric absorbance reading at 260nm equal to 1.

The DNA amount obtained in different isolation methods were found to be highly significantly different (Table 3). However, amount of DNA recovered in the same method was statistically similar. This indicates the accuracy of sample preparation during isolation of DNA using different methods. The highest amount of DNA was from the Method III which was highly significantly different from the DNA amount isolated from Methods I and II.

Table 1. Comparative statement of different DNA isolation methods in sugarcane.

Method-I	Method-II	Method-III
Meristem cylinder of sugarcane was cut into 10mm pieces. 1.5g of sample was placed in 50 ml Falcon tube.	The fresh leaf samples were cut into 1cm strips. Placed 2.0 g of sample in a autoclaved polypropylene bag.	The meristem cylinder was cut into 1cm strips. Placed 2.0 g of sample in an autoclaved polypropylene bag.
6 ml of extraction buffer was added (4 ml/g $^{-1}$fresh tissue)	16 ml of extraction buffer was added (8 mlg^{-1} fresh tissue).	4 ml of extraction buffer was added (2 mlg^{-1} fresh tissue).
Ultra-Turax was used for homogenization.	Mortar and pestle was used for grinding.	Mortar and pestle was used for grinding.
-	The extractant was collected by filtration.	The extractant was collected by filtration.
-	7.5 ml of extractant was poured into a 50 ml Falcon tube.	500 µl of extractant was poured into 2 ml eppendorf tube.
1.5 ml of 5% SDS, 1.5 ml of 10% PVP and 1.5 ml of 20% CTAB were added and mixed well by inversion.	2 ml of 5% SDS, 2 ml of 10% PVP and 2 ml of 20% CTAB were added and mixed well by inversion.	150 µl of 5% SDS, 150 µl of 10% PVP and 150 µl of 20% CTAB were added and mixed well by inversion.
Incubation was done for 30–60 min at 65 ^{0}C in a water bath.	Incubation was done for 30–60 min at 65 ^{0}C in a water bath.	Incubation was done for 30–60 min at 65 ^{0}C in a water bath.

Method-I	Method-II	Method-III
Samples were mixed by inversion 3–4 times during incubation.	Samples were mixed by inversion 3–4 times during incubation.	Samples were mixed by inversion 3–4 times during incubation.
Samples were taken from the water bath and cooled down to room temperature.	Samples were taken from the water bath and cooled down to room temperature.	Samples were taken from the water bath and cooled down to room temperature.
An equal volume of 25:24:1 phenol:chloroform:isoamyl alcohol was added and mixed by inversion.	An equal volume of 25:24:1 phenol:chloroform:isoamyl alcohol was added and mixed by inversion.	An equal volume of 25:24:1 phenol:chloroform:isoamyl alcohol was added and mixed by inversion.
Centrifuged the tubes at 3000g for 10 min at 4 ^{0}C.	Centrifuged the tubes at 3000g for 10 min at 4 ^{0}C.	Centrifuged the tubes at 10,000 rpm for 35 mins at room temperature.
The aqueous phase was recovered and transferred to a fresh ice cold 50 ml Falcon tube.	The aqueous phase was recovered and transferred to a fresh ice cold 50 ml Falcon tube.	Aqueous phase (about 800 µl) was recovered and transferred to a fresh ice cold 2 ml eppendorf tube.
An equal volume of ice cold isopropanol was added followed by 1.5 ml of 6 M NaCl.	An equal volume of ice cold isopropanol was added followed by 2 mL of 6 M NaCl.	An equal volume of ice cold isopropanol was added followed by 150 µl of 6 M NaCl.
Incubated at - 20 ^{0}C for at least 1 h.	Incubated at - 20 ^{0}C for at least 1 h.	Incubated at - 20 ^{0}C for at least 1 h.
-	-	Centrifuged at 10,000 rpm for 20 min at room temperature.
-	After incubation the solution was distributed to 1.5 ml eppendorf tube 1 ml in each.	Decanted the upper solution carefully by using adjustable micropipette.
4 ml of 70% ethanol was added and mixed properly by inversion.	300 µl of 70% ethanol was added and mixed properly by inversion.	70% ice cold ethanol about 2.5 times of the solution was added and mixed properly by inversion.
Centrifuged at 2,500 rpm for 3 min at room temperature.	Centrifuged at 10000 rpm for 1 min at room temperature.	Centrifuged at 10,000 rpm for 10 min at room temperature.
After DNA pellet formation the solution was discarded completely.	After DNA pellet formation the solution was discarded completely.	After DNA pellet formation the solution was discarded completely.
70% ethanol was added up to the slant of the tube. If have no time to continue work may be stored it at 4-6 ^{0}C for following days.	70% ethanol was added up to the slant of the tube. If have no time to continue work may be stored it at 4-6 ^{0}C for following days.	70% ethanol was added up to the slant of the tube. If have no time to continue work may be stored it at 4-6 ^{0}C for following days.
The 70% ethanol was discarded from the tube carefully that the DNA pellet remain constant and undisturbed.	The 70% ethanol was discarded from the tube carefully that the DNA pellet remain constant and undisturbed.	The 70% ethanol was discarded from the tube carefully that the DNA pellet remain constant and undisturbed.
DNA pellet was air dried	DNA pellet was air dried.	DNA pellet was air dried.
The DNA pellet was re-suspended in 2–3 ml of TE buffer.	DNA pellet was re-suspended in 50 µl of TE buffer.	DNA pellet was re-suspended in 50 µl of TE buffer.
Stored at –20 ^{0}C	Stored at –20 ^{0}C	Stored at –20 ^{0}C

Table 2. Ratios of spectrophotometric absorption readings at 260 nm and 280 nm of different samples in different isolation methods for quality determination of DNA.

Isolation Method	Sample No.	Absorbance Reading at 260 nm	Absorbance Reading at 280 nm	A260/A280 Ratio
Method-I	01	0.033	0.018	1.833
	02	0.031	0.018	1.722
	03	0.025	0.011	2.272
	04	0.029	0.018	1.611
	05	0.031	0.018	1.722
	06	0.028	0.018	1.555
Method-II	01	0.039	0.027	1.444
	02	0.029	0.030	0.966
	03	0.028	0.027	1.037
	04	0.039	0.030	1.30
	05	0.029	0.027	1.074
	06	0.039	0.032	1.218
Method-III	01	0.082	0.045	1.822
	02	0.092	0.054	1.703
	03	0.082	0.052	1.576
	04	0.092	0.045	2.044
	05	0.082	0.054	1.518
	06	0.092	0.052	1.769

Table 3. Spectrophometric absorption readings and concentrations of different DNA samples in different isolation methods.

Isolation Method	Sample No	Absorbance Reading at 260 nm	Concentration of DNA(ng.µl^{-1})	Average concentration(ng.µl^{-1})
Method-I	01	0.033	1650	1475.00b*
	02	0.031	1550	
	03	0.025	1250	
	04	0.029	1450	
	05	0.031	1550	
	06	0.028	1400	
Method-II	01	0.039	1950	1691.67b
	02	0.029	1450	
	03	0.028	1400	
	04	0.039	1950	
	05	0.029	1450	
	06	0.039	1950	
Method-III	01	0.082	4100	4350.00a
	02	0.092	4600	
	03	0.082	4100	
	04	0.092	4600	
	05	0.082	4100	
	06	0.092	4600	

* Figures followed by different letters in a column are different as per Duncan's New Multiple Range Test (DMRT) at p-0.01.

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