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Evaluation of Different Methods for Total DNA Extraction from Sago Pith Residue

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ABSTRACT

Direct extraction of DNA from the environment has become major importance for molecular analyses for the study of microbial communities in soil and other decomposing agrowaste. The presence of humic substances in Sago pith residue not only results in low DNA quality but also can lead to PCR amplification inhibition which may hinder most molecular studies. Many of the published protocols have been found to be unsuitable to obtained high amount of yield and low of humic acids contamination. This study presents the evaluation of three different methods for extracting total DNA from Sago pith residue. The methods evaluated were enzymatic lysis, glass bead homogenization and freeze-thaw treatment. Each method were evaluated by 260/280 nm absorbance ratio for protein contamination, 260/230 nm absorbance ratio for other contaminants and PCR amplification for molecular work suitability. Among the three methods, freeze-thaw treatment provided the highest yield of DNA, 5.06±0.01 µg/g of Sago pith residue. Nevertheless, all three methods resulted in poor DNA quality which could be used for PCR amplification. Additional steps of agarose electrophoresis and silica column purification were found to be effective for increasing the quality of the extracted DNA and were validated by positive PCR amplifications.

INTRODUCTION

Most microorganisms from environmental samples are difficult to be cultured. Only a small proportion of soil microorganisms are culturable on standard media [1]. For this reason, there are obstacles in understanding microbial ecology and diversity [2]. Isolation of bacterial nucleic acids from natural environments has become a useful tool to identify bacteria that cannot be cultured [3,4], to determine species of selected bacteria or genes under indigenous conditions [5], and to reveal genotypic diversity and its change in microbial ecosystems [6]. Environmental samples such as soils and composting agrowaste present some of the most difficult challenges to the development of suitable extraction and purification procedures. Most DNA extraction methods produced low DNA yield. Direct extraction of total DNA always results in co-extraction of other organic components, mainly humic acids or other organic substances, which negatively interfere with DNA transforming and detecting processes [1,4,5]. It has been reported that those substances inhibit restriction endonucleases [6,7] and Taq polymerase, the key enzyme of the polymerase chain reaction (PCR), and decrease efficiencies in DNA-DNA hybridizations [8]. This study were set to evaluate several DNA extraction methods in order to develop an effective DNA extraction method for extraction with and without further purification for production of higher DNA yields and less humic acid contaminations for PCR amplification.

MATERIALS AND METHODS

Sampling

Sago pith residue samples were collected from Ladang Dalat, Sago plantation in Mukah region. Samples were obtained between 5-10 cm in depth of Sago pith residue. Samples were maintained at 4 °C until use.

DNA extraction using enzymatic lysis

Extraction buffer (20 ml of 100 mM Tris-HCl [pH 8.0], 100 mM sodium EDTA [pH 8.0], 1.5 M NaCl) was mixed with 10 g (wet weight) of residue. 0.5 mg of proteinase K were added and

incubated at 37 °C for 30 min with shaking at 180 rpm. Three ml of 20% w/w sodium dodecyl sulfate (SDS) was added and samples were incubated at 65 °C for 90 min. Supernatant was collected after centrifugation at 6000 rpm for 10 min at room temperature. Next, same volume of 30% w/w PEG, 1.6 M NaCl solution was added and incubated at room temperature overnight [9]. The supernatant was recollected after centrifugation at 10,000 rpm for 20 min. Equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added and centrifuged at 5000 rpm for 5 min. Aqueous phase was collected and centrifuged with equal volume of chloroform:isoamyl alcohol (24:1) at 5000 rpm for 5 min. Aqueous phase was recollected and incubated with 0.6 volume of isopropanol for 2 hours. Sample was centrifuged at 13000 rpm for 15 min. The pellet was suspended in TE buffer and stored at -21 °C for further use.

DNA extraction using beads beating

Twenty ml of extraction buffer was mixed with 10 g (wet weight) of pith residue. Ten g of 0.1 mm glass beads (Scientific Industries, Inc, USA) were added and vortexed for 30 min. Extraction was continued as per enzymatic lysis method.

DNA extraction using freeze thaw

Twenty ml of extraction buffer was mixed with 10 g (wet weight) of pith residue. Proteinase K (0.5 mg) were added and incubated at 37°C for 30 min with shaking at 180rpm. Three ml of 20% SDS was added and samples were incubated at 65°C for 90 min. Next, 3 cycles of freeze thawing were perfomed. Extraction was continued as per enzymatic lysis method [10].

DNA purification

The extracted DNA was purified using Promega Gel clean up system. Following electrophoresis using 1% w/w agarose gel, DNA bands from gel was excised and placed in 1.5 ml microcentrifuge tube. Membrane binding solution was added 10 µl per 10 mg of gel slice and incubated in 50-65 °C until gel completely dissolved. Dissolved gel mixture was transferred into the mini column assembly and was centrifuged at 16,000 rpm for 1 min. Next, the flow through was discarded and 700 µl membrane wash solution was added into the mini column and centrifuged at 16,000 rpm for 1 min. Five hundred µl of membrane wash solution was added and recentrifuged. The mini column was inserted into new collection tube and 100 µl of nuclease free water was added into mini column. Then, it was centrifuged at 16,000 rpm for 1 min. Purified DNA samples were stored at -21 °C.

DNA yield and quality evaluation

To evaluate the purity of the extracted DNA, absorbance ratios at 260/230 nm (DNA / other contaminants) and 260/280 nm (DNA / protein) were determined using Halo RB-10 UV/Vis ratio beam spectrophotometer (Dynamica Scientific Ltd, UK) (Table 1). DNA concentrations were determined by measuring the absorbance at 260nm. The DNA concentrations and yields were calculated as follow:

DNA Concentration (µg/ml) = A_{260} reading × dilution factor × 50µg/ml

DNA yield (µg) = DNA concentration × total sample volume (ml)

Polymerase Chain Reaction (PCR)

The PCR amplification mixture consisted of approximately 100 ng of genomic DNA, 25 pmol of each primer, 2.5 mM $MgCl_2$, 0.225 mM of each dNTP (deoxynucleotide triphosphate) and 1U *Taq* DNA polymerase (Promega, USA). The general primer pair 1492r (5'TACGGYTACCTTGTTACGACTT3') and 27f (5'AGAGTTTGATCMTGGCTCAG3') were used. The following thermal cycle was performed: 94 ºC 3 min (1 cycle), 94 ºC 1 min, 55 ºC 1min, 72 ºC 2 min (30 cycles), 72 ºC 5 min (1 cycle).

Gel electrophoresis

Genomic DNA and pcr amplification reaction were analysed on 1% w/v agarose gels cast and run in TAE buffer (pH 8.0). Gels were stained with ethidium bromide and photographed using transmitted UV light. A 1kb marker (GeneRuler, Thermo Scientific, USA) was included on every gel.

RESULTS AND DISCUSSION

In this study, three different approaches were employed for total DNA extraction from Sago pith residue. All 3 methods were evaluated based on their purity, integrity, total yield and the suitability for downstream molecular procedures. Based on the absorbance ratio at 260/280 nm, all three methods showed DNA with ratio below than the ideal 1.8 (Table 1). This may indicate contamination of protein or other substances which may interfere with other downstream application. Absorbance ratios at 260/280 nm were used as an indicator for other contaminants particularly humic substances.

Table 1. Comparison of DNA purity and yield using different extraction methods.

Method	A_{260}/A_{230}	A_{260}/A_{280}	DNA concentration (µg/ml)	DNA yield (µg/g)
Enzymatic lysis	0.71±0.004	1.14±0.01	498.3±1.3	4.98±0.013
Glass beads homogenization	0.92±0.04	1.30±0.005	476.6±0.68	4.76±0.06
Freeze-thaw	0.99±0.009	1.32±0.001	506.6±1.3	5.06±0.01
Enzyme lysis + silica column purification	1.93±0.07	1.79±0.07	146.6±1.3	1.46±0.013
Bead beating + silica column purification	2.04±0.06	1.70±0.06	152.6±0.13	1.52±0.001
Freeze-thaw + silica column purification	2.38±0.07	1.77±0.06	138.3±0.6	1.38±0.006

The humic substances have similar size and charge characteristics to DNA resulting in their copurification [11], evident by the extractions being brown in colour. Organic matter is the major source of inhibitors that may be co-extracted with the microbial DNA. In particular, humic acids cause major problem and will interfere in enzymatic manipulations of DNA [12,13]. DNA polymerases found to be inhibited by as little as 1 µl of undiluted humic-acid-like extract, regardless of the amount of DNA present [14]. Humic contaminants also interfere in DNA quantitation since they exhibit absorbance at both 230nm and at 260nm [15], the later used to quantitate DNA. The purity of the extracted DNA varied as determined by the ratio of A_{260} to A_{280} and A_{260} to A_{230} (Table 1). A high 260/230 ratio (~2.0) is indicative of pure DNA, while a low ratio is indicative of humic acid contamination and a high 260/280 ratio (~1.8) is indicative of pure DNA, while a low ratio is indicative of protein contamination.

In all cases, DNA samples were of low purity. After all DNA samples were subjected to 1% agarose electrophoresis and silica spin column purification, contamination levels showed different results toward the three methods. All of the methods

showed significant improvements indicated by 260/230 ratios near to 2.0 and 260/280 ratios near to 1.8 (Table 1). The removal of humic substances and other contaminants were also assessed by PCR. Prior to 1% agarose electrophoresis and silica spin column purification, no amplification was observed while all purified DNA samples showed expected band of 1.5 kbp amplicon (**Fig.** 1).

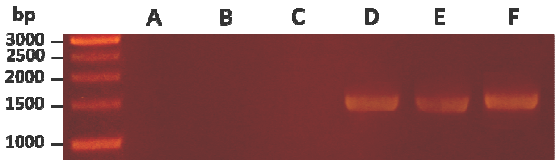

Fig. 1. Agarose gel electrophoresis of polymerase chain reaction (PCR) using total DNA extraction from different methods as templates. Lanes: A. Enzymatic lysis method; B. Glass beads homogenization; C. Freeze-thaw treatment; D. Enzymatic lysis method with silica column purification; E. Glass beads homogenization with silica column purification; F. Freeze-thaw treatment with silica column purification.

Results from the 1% agarose gel electrophoresis also showed that all 3 methods evaluated maintained high levels of integrity of DNA samples (**Fig.** 2). However, the integrity of DNA samples reduced significantly after agarose electrophoresis and silica spin column purification with DNA samples from freeze-thaw treatment and purification showing the highest level of degradation or DNA shearing. Consideration regarding DNA shearing is needed depending on the type of downstream molecular application. Noteworthy from this result, despite mechanical shearing by glass beads and vortexing, DNA sample extracted using glass beads homogenization method remained reasonable intact when compared with other methods.

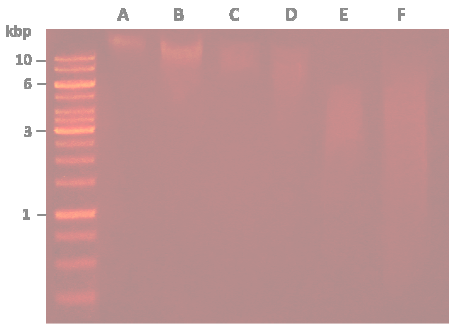

Fig. 2. Agarose gel electrophoresis of total DNA extraction using different methods. Lanes: A. Enzymatic lysis method; B. Glass beads homogenization; C. Freeze-thaw treatment; D. Enzymatic lysis method with silica column purification; E. Glass beads homogenization with silica column purification; F. Freeze-thaw treatment with silica column purification. Each lane contains 100 ng of DNA.

Yield of extracted DNA from Sago pith residue using all 3 methods were relatively close to each other even for the results before additional purification steps. There are several explanation for these yields (Table 1). Firstly, the DNA concentration prior to electrophoreis and purification steps may be inaccurate and unreliable due to heavy contamination by humic acid. Thus, the numbers shown as concentrations may be overestimation of actual concentrations. Secondly, for DNA concentrations post-purification steps, the silica absorption capacity of spin columns may be acting as the limiting factor. Specifications from manufacturer stated that each column could absorb as high as 40 µg of DNA. In this study, however, all three methods eluted 15 µg of DNA per spin column.

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