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Orignal Article

THE EFFICIENT DNA EXTRACTION PROTOCOL FOR SINGLE CELL MICROALGA (*COCCOMYXA* SP. SUA001)

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Abstract: The first step in molecular biology research is to isolate high-quality DNA. The microalga Coccomyxa possess thick cell wall and hence to extract DNA material from those cells is a challenging task. Very limited protocols are available for such single-cell microalga. In this research, we developed a simple, efficient, and high throughput DNA extraction protocol from single-cell Coccomyxa species. The spectrophotometric and gel electrophoresis analyses revealed that the genomic DNA isolated using this approach had a high yield and good quality. The quality of extracted DNA was confirmed using PCR amplification for 18S rRNA gene. This approach is simple and efficient for standard DNA extraction from single-cell microalga.

Keywords: ***Coccomyxa*** species, Single-cell microalgae DNA extraction

INTRODUCTION

The green photosynthetic microalga ***Coccomyxa*** species is a member of the Trebouxiophyceae class. Since Schmidle's 1901 [1], in description of the genus ***Coccomyxa,*** more than 50 species have been named based on morphology and life cycle [2,3]. Among green coccoid algae, the genus ***Coccomyxa*** is one of the most tangled. It is distinguished by its single cell, relatively small size (6-14 x 3-6 µm), variable spherical to oval cell morphology, parietal chloroplast without a pyrenoid, and absence of flagellated phases [4,5]. ***Coccomyxa*** is widely distributed and known for its ecological adaptability. It can be found as a free-living organism in terrestrial green biofilms, as soil algae linked with mosses [1-6] and as a planktonic organism in limnic habitats with higher plants and the ability to infest marine mussels [7-9]. ***Coccomyxa*** can even be found as a contaminant in research labs, in various chemicals or distilled water [10] or in a nuclear power plant in cooling water [11].

Microalgal cells are typically small (often <10µm), have thick walls, and are rich in molecules such as both chlorophyll a and b, β-carotene, xanthophylls, cellulose, and starch [12] which have a higher influence on DNA isolation, affecting cell lysis and downstream applications such as PCR amplification [13,14]. Methods for improving the quality of extracted genomic DNA have focused on purity and yield, as these characteristics have the greatest influence on the success of downstream applications (PCR, hybridization, and activities of restriction enzymes). The perfect extraction methods depending upon the cetyltrimethyl ammonium bromide (CTAB) extraction process [15] or using species appropriate extraction buffers can improve sample purity [6-17]. Also, a number of commercially available kits containing unique buffers or columns have also been created to overcome the challenge of isolating high-purity DNA from plants and green microalgae [13]. In this study, we have not used the harsher chemical, mechanical and enzymatic treatments such as

guanidine isothiocyanate, sarcosyl, sonication, bead-beating, or lysozyme, which increase DNA fragmentation [25,26].

Here, we present a highly scalable DNA extraction protocol specifically designed for extracting high-quality DNA suitable for downstream application in single-cell microalga ***Coccomyxa*** sp. SUA001.

MATERIALS AND METHODS

Sample collection: Microalga **(*Coccomyxa*** sp. SUA001) was obtained from the VIRSACO institute, Veko house, Rajkot, Gujarat. It is growing in the culture of MS media with macro and micronutrients at room temperature under the 12h light/dark cycles. It is a free-living, single-cell of relatively small in size (5-10 x 3-5 µm), and spherical to oval in shape (Fig.1).

DNA extraction protocol: DNA was extracted from 50 ml of liquid culture of microalgae from the exponential phase. Cells were harvested by centrifugation at 10,000 g for 5 min and pellets were transferred to eppendorf tubes. Thereafter, 1 ml of lysis buffer (Urea 4 M; Tris-HCl 0.2 M, pH 7.4; NaCl 20 mM and EDTA 0.2 M) and 50 µl proteinase K (stock solution of 20 mg/ml) was added to the pellet and mixed immediately by pipetting. This mixture was incubated for 1h at 55°C in waterbath and mixed by pipetting every 10- 15 min. Thereafter, 2 ml of prewarmed (55°C) DNA extraction buffer (CTAB 3%; NaCl 1.4 M; EDTA 20 mM; Tris-HCl 0.1 M, pH 8.0; SDS 1% and β-mercaptoethanol 1%) was added, mixed gently by inverting the tubes and incubated at 55°C for another 1 h. During incubation, eppendorf tubes were gently inverted 4-5 times at every 10 min to mix the solution. After this step the mixture was divided into two fractions in eppendorf tubes, allowed to cool, and 2 volume of chloroform: isoamyl alcohol (24:1 v/v) was added to it and mixed by gentle inversion until a white emulsion appeared. After centrifugation at 10,000 g for 5 min, 500 µl from the upper phase was transferred to a new eppendorf tube and extracted with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1). After centrifugation at 10,000 g for 5 min, the upper aqueous phase was transferred to a new tube, 4µl RNase (stock solution of 2 mg/ml) was added and incubated at 37 °C for 1 h, then for 20 min it was kept at -20 °C. The supernatant was transferred to a new tube and one volume of isopropanol was added to it, mixed once by inversion, and transferred to -20°C for 1 h. After 1 h incubation suspensions were centrifuged at 10,000 g for 3 min and the supernatant was discarded. Thereafter, pellet was washed with 70% ice-cold ethanol (500 µl) and after evaporating the residual ethanol dry pellets were dissolved in 100 µl of TE buffer (10mM Tris- HCl (pH 8.0), 1mM EDTA). The extracted DNA was stored at 4 °C.

DNA quantification and agarose gel electrophoresis: The genomic DNA concentration was quantified by spectrophotometer (Analytik Jena, SPACORD 200). The purity of genomic DNA was checked by the ratio between absorption at 260 and 280 nm. The quantity of the sample's DNA was calculated using the formula given by Sambrook and Russell [19]. The integrity of the extracted genomic DNA was tested by 1% agarose gel electrophoresis.

18S rRNA gene amplification and agarose gel electrophoresis: DNA was amplified by using 18S rRNA primers as follows: Chloro R- TCAACAA-ATCATAAAGATATTGG and Chloro F-ACTTCTG-GATGTCCAAAAAAYCA [20]. The PCR amplification was conducted using 25µl of the reaction mixture which contains genomic DNA, 10X buffer, 25mM MgCl2, 10mM dNTPs, 18S rRNA forward and reverse primers, Taq DNA polymerase, and mili Q to a final volume of 25 µl. The PCR cycles were: 1 cycle of denaturation at 94°C for 2 mins, 30 cycles of denaturation at 94°C for 1 min, annealing at 57°C for 18S rRNA, and final extension at 72°C for 7 mins, and final sample hold on 4°C. Amplified DNA was evaluated using 2% agarose gel electrophoresis.

RESULTS AND DISCUSSION

In the present study ***Coccomyxa*** sp. SUA001, was found to be morphologically similar to other ***Coccomyxa*** sp. reported in the literature (Fig.1). We have collected this alga from the ***in vitro*** contaminated plant tissue culture media. It is growing in the culture of MS media with macro- and micronutrients at room temperature. We have isolated the genomic DNA from the ***Coccomyxa*** sp. SUA001 and the sharp band of the genomic DNA was found in 1% agarose gel electrophoresis (Fig. 2). DNA purity and concentration were measured in a UV spectrophotometer. The purity of DNA was observed as 260/280 nm ratio. The purity was 1.83 and the concentration was 130.89 µg/mL observed. The DNA was considered pure if

the ratio between 260 and 280 nm is between 1.8 and 2.0. A ratio below 1.6 is typical for protein contamination while the above 2.0 is characteristic of RNA contamination. On the basis of DNA concentration and purity in ***Coccomyxa*** sp. protocol was better than other tested protocols. Using the 18S rRNA primer set a 500bp DNA fragment was amplified. Figure 3 shows 2% agarose gel electrophoresis of amplified 500bp 18S rRNA products from ***Coccomyxa*** sp. SUA001.

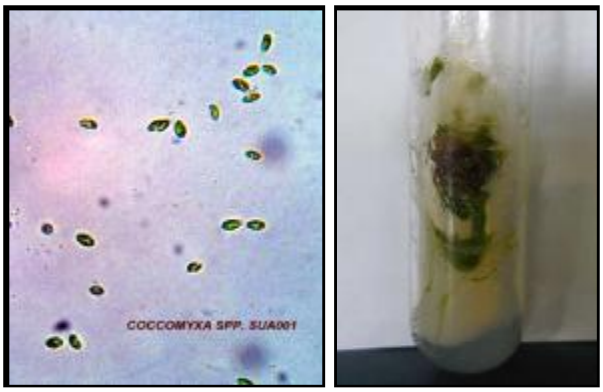

Fig. 1: (A) Light microscopy of the **C*occomyxa*** sp. SUA001 **(B)** Contaminant culture media

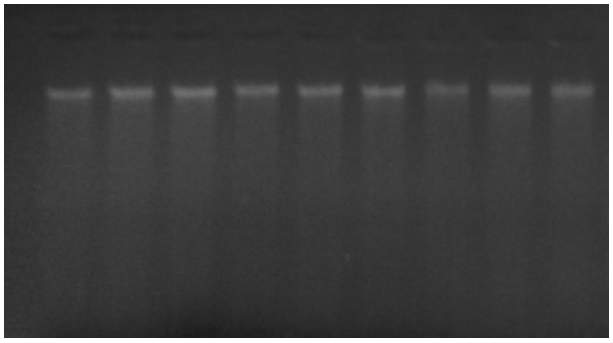

Fig. 2: 1% Agarose gel electrophoresis of genomic DNA from ***Coccomyxa*** sp. SUA001

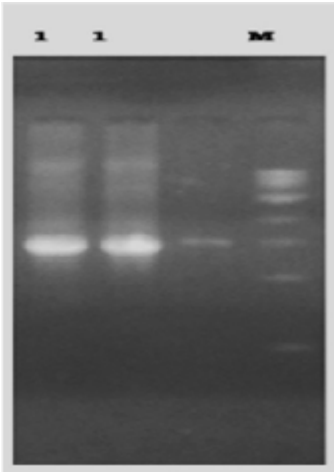

Fig. 3: 2% Agarose gel electrophoresis of amplified 500bp 18S rRNA products from ***Coccomyxa*** sp. SUA001

In this protocol, we used the lysis buffer and extraction buffer. In lysis buffer, we used the 4M urea, which is a chaotropic agent, and its role is to remove DNA from the histones and denature other proteins and promote more stability to the system [17-22]. In extraction buffer, CTAB (cetyl trimethyl ammonium bromide) is a cationic surfactant useful for the isolation of DNA from tissues containing high amounts of polysaccharides. The CTAB binds the polysaccharides and removes them from the solution [15-21]. Whereas, Sodium dodecyl sulphate (SDS) is an anionic detergent that can solubilize the proteins and lipids that form the membranes. This help to rupture cell membrane barriers, and SDS helps release the DNA from histones and other DNA-binding proteins by denaturing them. Hence the urea along with SDS helps to get a higher concentration of DNA [22,23]. The proteinase K enzyme degrades proteins into free amino acids upon incubation. Proteinase K, a subtilizing related serine protease, is not inhibited by either EDTA or β-mercaptoethanol. It is stable across a wide pH range (6.5-9.5), and the addition of SDS increases its activity seven fold [24]. The addition of the saturated phenol in the form of P: C: I step also helped to remove polysaccharides from the cell lysate. Figure 2 indicates contamination free DNA obtained by this protocol could be as a result of the RNase treatment. It was reported that the absence of RNA is evidence of good-quality genomic DNA [19].

The advantages of this protocol are: 1. There is no use of liquid nitrogen as it is single-cell. 2. There is no use of harsher chemical, mechanical and enzymatic treatments such as guanidine isothiocyanate, sarcosyl, sonication, bead-beating, or lysozymes which, increase DNA fragmentation [25,26]. This protocol is completed within five hours. Further, high purity and high yield DNA tested for 18S rRNA gene showed good amplification (Figure 3). Thus this protocol will be useful for the downstream applications like PCR, hybridization, and activities of restriction enzymes. This protocol may also be useful for preparing genomic DNA from other single-cell algae.

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