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Optimization of Two Different Modified Methods for High Quality and Quantity Extraction of DNA from Microalgae

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ABSTRACT

DNA extraction is one of the first tasks in the genomic study, and finding an effective protocol for the isolation of DNA is essential in genetic engineering and most microbiology studies. DNA extraction from microalgae has always been a challenge for microbiologists due to their tough and complex cell walls and inhibitory substances in their cellular structures. Also, inefficient or differential DNA extraction of microalgae community members can lead to bias in downstream community analysis; therefore, pure DNA extraction for molecular studies is of particular importance. In this study, the efficacy and experimental variability of two modified protocols (Phenol/Chloroform's method, and dellaporta DNA extraction's method) for total genomic DNA extraction from four microalgae were studied. The selected microalgae for this study were *Dunaliella salina*, *Haematococcus pluvialis*, *Nannochloropsis oculata*, and *Spirulina platensis*. The concentration of DNA was measured using Nanodrop and agarose gel electrophoresis. Both modified and optimized methods used in this study showed reliable and effective results and can be recommended for future studies; However, the results indicated that the optimized phenol/Chloroform's method is preferable to the dellaporta DNA extraction method. The highest obtained amount of DNA was related to *Dunaliella salina* (815.1 and 506.8 ng/µL) in phenol/Chloroform and dellaporta DNA extraction's method, respectively; and the lowest amount was related to *Spirulina platensis* with concentration 112.4 ng/µL (in Phenol/Chloroform's method) and 30.5 ng/µL in dellaporta DNA extraction's method. The extracted DNA was free from protein and polyphenolic/ polysaccharide compounds, therefore, they are recommended for all molecular studies on Microalgae.

Introduction

In recent years, scientific and commercial interest in microalgae has been increased progressively; because microalgae can be a valuable and sustainable global biomass for the production of biofuels, fertilizers and animal feed and etc. (Christenson and Sims, 2011). Microalgae in waste-stabilization ponds also play an important role in the uptake of nitrogen and oxygen tides for low-cost wastewater treatment by organic carbon degradating heterotrophic bacteria. In fact, key catalysts in waste consolidation ponds include microalgae, which control many of the processes in these ponds. Despite the important role of microalgae in the removal of nitrogen from wastewater, there is still insufficient knowledge of their biology and biodiversity (Camargo Valero and Mara, 2007, Curtis *et al.*, 2003). Also, microalgae are one of the most important photosynthetic organisms on earth, which are the main producers in the food chain and are considered as one of the food sources for humans and animals (Martin and Alexander, 2018). In industry and medicine, they are used as important biomass (Michael, 2018). So far, molecular studies on microalgae have included DNA barcoding (Beom-Ho *et al.*, 2014), building a microsatellite library (John *et al.*, 2010), and whole-genome sequencing (Crysten and Sabeeha, 2019). For these molecular methods, different types of DNA extraction methods have been used, such as: SDS (sodium dodecyl sulfate) method (Marmur, 1961; Takashi *et al.*, 2012), CTAB (cetyltrimethylammonium bromide) method (Doyle and Doyle, 1990), high salt low pH method (Guillemaut and Drouard, 1992), Triton x-100 method (Tomasz *et al.*, 2017), Chelex -100 method (Utkarsha *et al.*, 2018), salt method (Aljanabi and Martinez, 1997) and NaOH method (Hill-Ambroz *et al.*, 2002), which each of these protocols has a different quality and quantity of extracted DNA (Kunyalung *et al.*, 2020).

Indeed, obtaining high-weight molecular materials like DNA or RNA to create a genome library or for cloning,

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hybridization, and simple PCR reactions can be a serious problem in many organisms. On the other hand, it is very difficult to separate DNA from plants and algae because of their hard walls (Doyle and Doyle, 1987). In addition, the method used for one plant or group of algae is not effective for other groups; because of the likely diversity of cell wall structure, storage, and secondary constituents in the wall, that makes it difficult to access their genome material, many of which Compounds inhibit the enzymatic activity involved in cell wall digestion (Huang *et al*., 2000). Extraction of DNA from seaweeds containing a large amount of sulfated polysaccharides in the cell wall and intercellular matrix, is complex and time-consuming. Most methods used to extract DNA from green algae (Meusnier *et al.*, 2004), red algae (Waittier *et al.*, 2000), and brown algae (Phillips *et al.*, 2001) require liquid nitrogen which in this case, the viscous soluble polysaccharides are released from the DNA and the genetic material is released by grinding algal material in liquid nitrogen (Brasch *et al*., 1981).

Obtaining a combination of high concentration and purity of DNA without any trace of contamination with proteins, polysaccharides and very few fragmented DNAs is an essential prerequisite for genetic engineering and DNA sequencing (Linnarsson, 2010, Healey *et al.*, 2014). The complexity of DNA separation from eukaryotic algae have been repeatedly reported (Maneeruttanarungroj and Incharoensakdi, 2016, Tear *et al.*, 2013, E. Eland *et al.*, 2012, Jagielski *et al.*, 2017) because of the unique cell wall structure that contains biological molecules such as algaenan and dinosporin (Domozych *et al.*, 2012, Jagielski *et al.*, 2017). In addition, algae always form unique compounds in the cell wall to adapt to their habitat, such as mucilage sheaths containing a compound that is tolerant to cell lysis (Popper *et al.*, 2014; Kunyalung *et al.*, 2020). For these reasons, optimizing the DNA extraction method for a variety of uses in algae has become a major challenge. The purpose of this study was to optimize two existing DNA isolation methods for sequencing and engineering work in subsequent generations for *Haematococcus*, *Nanochloropsis*, *Dunaliella* and Spirorulina microalgae. So far, no suitable protocol has been reported for extracting genetic material from several different genera of algae, which is probably due to the lack of a fast, efficient and low-cost method for extracting genomic DNA with high quality and quantity from algae.

Materials and Methods

2.1. Algal source preparation

Four different microalgae species, Dunaliella salina, Haematococcus pluvialis, Nanochloropsis oculata and Spirorulina platensis which had been originally isolated and stored at the Nutrition Research Center, Tabriz University of Medical Sciences, were used in this study. All four selected microalgae species are native to the Iran microflora and are widely used in many biotechnology, pharmaceutical, and industry researches. The used media with the specific culture conditions of each for 4 algal in this study are described in Table 1.

2.2. Cell growth condition

Microalgae cells of each species were picked from a single colony on special culture medium with agar and were grown in a 100 mL of liquid medium for a week at 28 °C with shaking (110 rpm) until the optical density at OD 600 reached 5. 10^6 cell/ml.

2.3. Genomic DNA extraction protocols

Two different common methods of DNA extraction - phenol/ Chloroform method and dellaporta DNA extraction - after various modifications and opitmization were evaluated for DNA extraction from selected Microalgae. For this purpose, at the first step, 10 ml of the liquid culture was centrifuged (10 min, 5000 rpm) separately then the pellets were washed in 1 ml of distilled water.

Table 1. Culture conditions of the Microalgae used in this study

Algae species	Culture medium	Dark: light cycle (h)	Temperature (oC)	Ref.
Dunaliella salina	Johnson medium	8:16	28	**(Johnson et al., 1968)**
Haematococcus pluvialis	BG-11 medium	8:16	28	**(Stanier et al., 1971)**
Nanochloropsis oculata	f/2 medium	8:16	28	**(Guillard, 1975)**
Spirorulina platensis	Zarrouk medium	8:16	28	**(Zarrouk, 1966)**

2.4. Method 1: Modified Extraction with phenol/chloroform

Briefly, the algal cells were suspended in extraction buffer (50 Mm Tris HCl, pH=8, 20 mM EDTA, 200 mM NaCl, 1% SDS). Then DNA was further extracted using phenol–chloroform–isoamyl alcohol (Phe/Chl/IAA, 25:24:1). This was done in two steps. The aqueous phase was transferred into a new 1.5 ml microtube, then the aqueous phase was treated with 2 µl RNase A (Pishgam, Iran, Tehran) for one hour, to remove RNA. DNA was then extracted twice with chloroform-isoamyl alcohol (Chl/IAA, 24:1). To precipitate the genomic DNA, 1 ml of absolute ethanol was added, the obtained pellet was washed with 70% EtOH, then dried and dissolved in 50 µL of distilled water.

2.5. Method 2: Modified Extraction by dellaporta method

This method has been originally used for DNA isolation from plant cells (L. Dellaporta et al., 1983) but with some modifications and optimization was used for DNA extraction from selected Microalgae in this study. At the first step, pellets obtained by centrifuging were immersed in 15 ml of extraction buffer (0.5 M EDTA pH 8.0, 1 M Tris pH 8.0, 4 M NaCl), with RNase A (50 µg/mL), and 1ml SDS 20%. After mixing by strong shaking, and incubating in a 65°C water bath for 10 min, potassium acetate was added then centrifuged at 5000rpm, then to the upper part 10 ml of isopropanol was added and incubated at -20 °C for 30 min. Subsequently, the tubes were centrifuged for15 min at 13,000 rpm. The supernatant was completely transferred into the new tube. Re-dissolve each DNA pellet with 0.7 mL EB2 buffer (0.5 M EDTA pH 8.0, 1 M Tris pH 8.0), then the floating part was precipitated by addition 3 M NaOAC and 500 µL Isopropanol then centrifugation for 10 min at 13000 rpm. Finally, the resulting pellet DNA was rinsed with ethanol (1 ml of 70%) then - after drying the pellet- the resulting DNA was dissolved in 50 µL of distilled water.

2.6. DNA quantification and purity

Nano-drop spectrophotometer (Thermo Fisher Scientific, USA) was used to determine the concentration of extracted DNA in all samples. Nucleic acid quality (indicated by the absorbance ratio at 260 nm/230 nm) and protein contamination rate (indicated by the absorbance ratio at 260 nm/280 nm) were evaluated with the Nano-drop.

2.7. DNA integrity

To evaluate the quality and integrity of collected DNA samples, about 5 µl of each sample, were loaded into 1% agarose gel. DNA marker was used to determine the size of the extracted DNA. Then in the gel tank -containing TBE buffer (Tris, Boric acid, EDTA)- the samples were electrophoresed for 90 min. The quality of the bands was compared with due attention to their sharpness and DNA smear.

3.Results and Discussion

3.1. DNA concentration and quality

Typically, the absorption ratio at 260 nm and 280 nm is a parameter for the presence of DNA in comparison to other pollutants that absorb waves at 280 nm. The highest quality of DNA is extracted at a ratio of 260/280 to above 1.80, which is suitable for downstream applications, the most important of which is sequencing (E. Eland et al., 2012). Determination of the quantity and quality of extracted DNA at a wavelength of 260 nm was performed on Nanodrop. The purity and polysaccharide contamination levels of DNA were determined by examining the absorbance at 260 nm wavelengths and 280 nm and 230 nm respectively. In the present study, in both methods, the extraction for all microalgae samples with the ratio of 260/280 was ≥1.8; which indicates the DNA samples are protein-free with high-quality specimens.

The qualitative and quantitative results of the extracted DNA for the two modified methods were listed in Tables 2 and 3. Based on the results, the phenol/chloroform method with a concentration greater than 112 ng/µl was preferable in the DNA yield.

As the results presented in tables 2 and 3, the concentrations range from high to low was Dunaliella salina, Haematococcus pluvialis, Nanochloropsis oculata, Spirorulina platensis, respectively. As in the phenol/ Chloroform method, the concentration were 815.1 ng/µl for Dunaliella salina, 287.6 ng/µl for Haematococcus pluvialis, 218.7 ng/µl for Nanochloropsis oculata and 112.4 ng/µl for Spirorulina platensis; and in the dellaporta,s protocol, the concentration were 506.8 ng/µl for Dunaliella salina, 227.6 ng/µl for Haematococcus pluvialis, 39.4 ng/µl for Nanochloropsis oculata and 30.5 ng/µl for Spirorulina platensis. Dunaliella salina was reported in both methods with the highest DNA concentration yield and Spirorulina platensis with the lowest yield.

Table 2. Extracted of DNA concentration extracted by phenol/chloroform method for four different algae

Algae	Absorbance		Concentration (ng/µL)
	260:280	260:230	
Dunaliella salina	1.85	1.10	815.1
Haematococcus pluvialis	1.82	0.54	287.6
Nanochloropsis oculata	2.33	1.16	218.7
Spirorulina platensis	2.05	0.50	112.4

Table 3. Extracted DNA concentration by Dellaporta method for four different microalgae

Algae	Absorbance		Concentration (ng/µL)
	260:280	260:230	
Dunaliella salina	1.80	0.88	506.8
Haematococcus pluvialis	1.96	1.68	227.6
Nanochloropsis oculata	2.32	0.57	39.4
Spirorulina platensis	2.11	0.62	30.5

3.2. DNA integrity

In most molecular studies and genetic diversity based on polymerase chain reaction, in order to obtain accurate and complete information, high quality DNA samples with the least contamination of polysaccharides, phenols, proteins, etc. are needed. In microalgae, DNA extraction is generally challenging due to the thick cell wall and high content of secondary metabolites (Zang *et al.*, 2013). Extraction of genomic DNA and then PCR reaction for molecular analysis in different species of algae is usually difficult due to the abundance of secondary metabolites (polyphenols, polysaccharides, etc.). Therefore, a method for high quality and quantity DNA extraction is needed that minimizes the waste of time, cost and energy in DNA extraction. So far, a number of protocols for extracting the genetic material from algae have been reported (Fawley and Fawley, 2004, Hwang *et al.*, 2010). In general, algae cell pellets obtained from centrifugation are solved in detergent buffers such as SDS to lyse the cell membrane, which is performed at least once in phenol/chloroform buffer and once in chloroform. In some cases, treatment with RNase performs to purify DNA from RNA. Samples then are precipitated by adding ethanol or isopropanol and are placed on ice or freezer. After the DNA pellet is precipitated by centrifugation, it usually suspends in deionized- water or TE buffer, and quality determination performs with a spectrophotometer at 260 nm (Wittkopp, 2018).

The aim of this study was to extract fast, easy and cheaper genomic DNA with high quality and quantity from all four mentioned microalgae based on two methods: Modified Extraction with phenol / chloroform and Modified Extraction by dellaporta's method.

In the phenol /chloroform protocol described in this study, two steps of extraction with phenol / chloroform / isoamyl alcohol buffer and two steps of chloroform/ isoamyl alcohol extraction buffer were used and between the two steps of extraction buffer, one treatment stage with RNase was performed. The role of isoamyl alcohol in organic solvents was to inhibit interphase cleavage and stabilization, which involves high concentrations of coagulated proteins. The role of phenol in extraction is to remove protein from nucleic acid. However, the main function of chloroform is to protect DNA when extracting genomic material. Chloroform plays an important role in increasing the efficiency of phenol in denaturing protein. Chloroform promotes proper separation of the organic phase and the aqueous phase and effectively protects DNA in the aqueous phase. An important point of this protocol was high quality and quantitative DNA extraction that was suitable for gene cloning and sequencing.

Several changes were made to the Dellaporta DNA extraction's protocol. In DNA extraction using this protocol, some steps such as using liquid nitrogen and Mercaptoethanol were omitted; but as can be seen in the results of DNA electrophoresis (Figure 2), DNA concentration was still high. This indicates that if the rest of the steps, such as preparing the buffer and adjusting the pH of the solution buffer are done carefully, the role of the two substances in the extraction is small and can be ignored. Also, quantitative and qualitative measurement of DNA electrophoresed in 1% agarose gel indicates the presence of sharp bands without smear, which indicates the purity of the extracted DNA (figure 1 and 2). Standard electrophoresis of DNA extracts showed that phenol/chloroform method had better results than dellaporta DNA extraction method. In phenol/chloroform method, *Dunaliella salina* and *Haematococcus pluvialis*

microalgae showed higher and clearer concentrations than *Nanochloropsis oculata, Spirorulina platensis* algae (Figure 1). Also, in the Dellaporta method, *Dunaliella* and *Haematococcus* showed a better band than two other algae (Figure 2).

According to the previous studies, the highest concentration of DNA extracted in the method proposed by Jagielski *et al*. (2017) for the microalga *Prototheca wickerhamii* 74.2 ng / µL, in the study by E. Eland *et al.* (2012) for freshwater eukaryotic microalgae 323 ng / µL, and in the study by Varela-Alvarez *et al.* (2006) for *Caulerpa taxifolia* 206 ng / µL, have been reported. However, the highest concentration of DNA extracted in the optimized methods in the present study was reported for *Dunaliella salina* (815.1 and 506.8 (ng / µL)) in phenol / Chloroform and dellaporta DNA extraction[s] method respectively which both were higher than all previous reports.

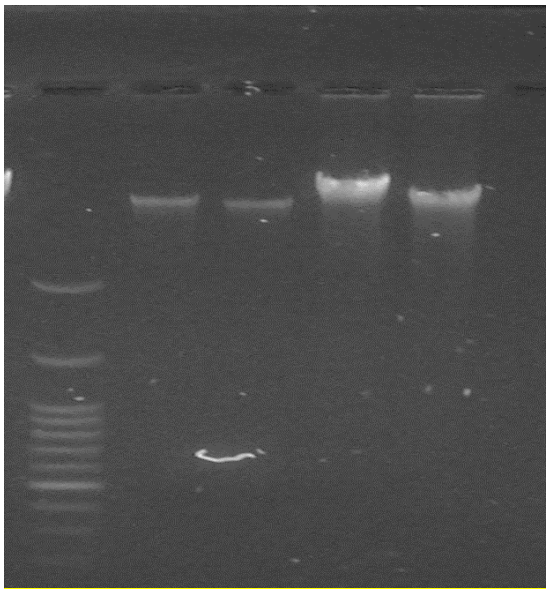


Figure 1. Standard 1% agarose electrophoresis gel; Evaluation of DNA integrity by phenol/ Chloroform extraction method for four different algae. lane M: GeneRuler 1500 bp DNA Ladder (CinnaGen, Cat. No. PR901644), lane1: Spirorulina platensis, lane2: Nanochloropsis oculata, lane3: Dunaliella salina, lane4: Haematococcus pluvialis.

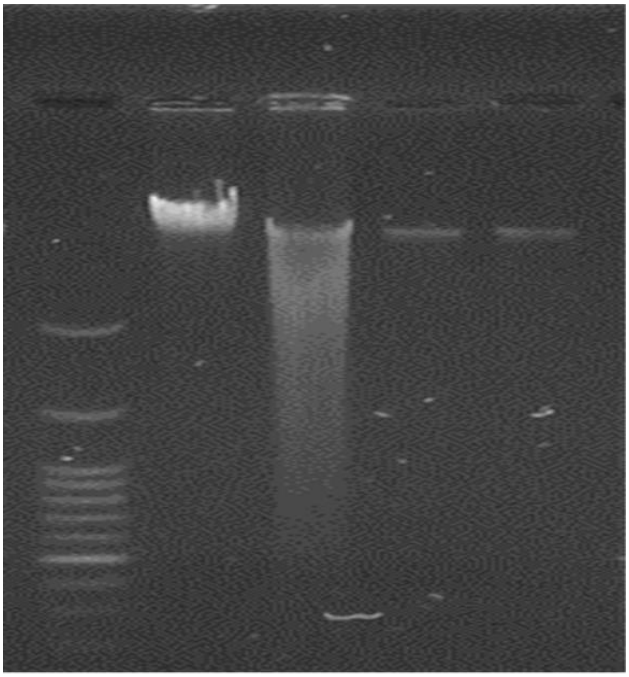


Figure 2. Analyses on 1% agarose gel of genomic DNA (5 µL) for four different algae by Dellaporta extraction method. lane M: GeneRuler 1500 bp DNA Ladder (CinnaGen, Cat. No. PR901644), lane1: Dunaliella salina, lane2: Haematococcus pluvialis, lane3: Nanochloropsis oculata, lane4: Spirorulina platensis

4. Conclusion

In the present study, two high-efficiency DNA isolation protocols were modified and optiied for green microalgae with high DNA yield. The methods used in the present study have eliminated the fundamental difficulty of many researchers in breaking down the hard wall of algae; and the other benefits of these two methods are the high concentration and quality of DNA present in various molecular genetic technologies. Protocol of phenol/chloroform has been shown to be superior to the Dellaporta extractions method for green microalgae. As the concentration of extracted DNA for the four algae in this method is significantly different from the Dellaporta,s method.

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