



## Improvement of DNA Extraction Protocols for *Nostochopsis* spp.

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### ABSTRACT

*Nostochopsis* spp. are a type of cyanobacteria which form mucilaginous balls. These cyanobacteria have a high content of polysaccharides, which makes it difficult to isolate their genomic DNA by the conventional method. In this research study, six protocols for improvement of DNA extraction from this cyanobacterium, including; crushing with glass beads and liquid nitrogen, washing with 3 M NaCl and crushing with glass beads, freezing-thawing, crushing with glass beads and sonication, crushing with liquid nitrogen and adding polyvinylpolypyrrolidone (PVPP), as well as crushing with glass beads and adding lysozymes, were compared. It was found that, these protocols could improve both the quantity and quality of the extracted genomic DNA. Crushing with liquid nitrogen and adding polyvinylpolypyrrolidone was found to be most suitable for *Nostochopsis* spp. genomic DNA extraction. This method could provide the highest yield of the extracted genomic DNA. Moreover, the genomic DNA obtained was highly cleaned of the contaminated proteins and polysaccharides and it was found to be a high quality DNA template for PCR. In addition, this extraction method was also effective for other high polysaccharide content cyanobacteria, such as *Nostoc commune* and *Phormidium* sp.

**Keywords:** cyanobacteria, DNA extraction, *Nostochopsis* spp.

### 1. INTRODUCTION

Cyanobacteria, cyanophytes or blue green algae are fascinating organisms. They are widely distributed in natural environments and are considered a major component of microbial populations in terrestrial and aquatic habitats worldwide [1]. They are also an interesting functional food source [2]. These microbes have also been reported to be rich sources of healthy nutrients such as proteins, carbohydrates, vitamins, minerals,

amino acids, and fatty acids. *Nostochopsis* (Lon, a common name in Thailand and Laos) is a filamentous cyanobacterium, normally found in the form of mucilaginous balls. The local people in northern Thailand, especially in Nan Province, consume it as a food substance and as a traditional form of medicine [3]. Local tribes of India have long used it as a dietary supplement [2]. This cyanobacterium is known in many

countries as a cosmopolitan species [4], but only a few investigations have reported on its genotypic information. However, the study on its genotype is considered rather difficult, because the isolation of genomic DNA from filamentous cyanobacteria often poses problems due to its branching patterns and additional surface structures, such as its mucilaginous sheath, S-layer, pili, slime, capsule, as well as other characteristics. [5]. In addition, it has a high content of polysaccharides of up to 49% w/w [3,6]. The polysaccharides, polyphenols and other secondary metabolites make it very difficult to isolate a satisfactory quality of DNA. These compounds bind tightly to nucleic acids during the isolation of DNA and interfere with other subsequent reactions [7]. Most genetic diversity studies use molecular tools, which are mainly based on a polymerase chain reaction (PCR) that requires DNA of good quality. Moreover, reports on the difficulties in isolating DNA of acceptable is common in high polysaccharide plants and many companies produce genomic DNA extraction kits for these plants. They are more convenient, easier and less toxic than chemical extraction methods, although the extraction of DNA through the use of these extraction kits has been successful in many plants with mucilaginous algae [8, 9]. However, this method has not worked with mucilaginous cyanobacteria, and particularly *Nostochopsis*. The lysis buffer and lysis condition in those kits are not sufficient to obtain good quality and a high quantity of DNA from these cyanobacteria. Therefore, many plant and cyanobacterial lysis methods have been developed [5,9,10,11,12 and 13]. However, the comparison between the efficiency of these methods has not yet been investigated. Consequently, this research was aimed to compare the pros and cons of the six DNA extraction protocols, which involve

physical and chemical means for the improvement of high quantity DNA isolation from high polysaccharide cyanobacteria, such as *Nostochopsis* spp., as well as other cyanobacteria.

## 2. MATERIALS AND METHODS

### 2.1 Cyanobacterial Strains

*Nostochopsis* sp. NT1 and *Nostochopsis* sp. NT2 were collected from the Nan River in Nan Province. Samples of *Nostochopsis* sp. CM3 were collected from a glasshouse of the Sirikit Botanical Garden, Chiang Mai. Samples of *Nostochopsis* sp. MP2 were collected from Khong Muang Canal, Mae Hong Son. *Nostochopsis* sp. TISTR8894 and *Nostoc commune* TISTR6290 were obtained from Thailand Institute of Scientific and Technological Research. *Phormidium* sp. AARLC009 was obtained from the stock culture of the Applied Algal Research Laboratory (AARL), Chiang Mai University. All cultures were maintained in liquid nitrogen-free BG-11 medium [14], at 24°C under continuous cool-white fluorescent lighting with a light intensity of 8.76  $\mu\text{mole.m}^{-2}.\text{s}^{-1}$ . Twenty-day old samples were used in each experiment.

### 2.2 DNA Extraction

In the conventional protocol of a Genomic DNA Mini Kit (Geneaid), 50 mg of fresh *Nostochopsis* NT2 was mixed with 400  $\mu\text{L}$  of GP1 lysis buffer and 5  $\mu\text{L}$  of RNase A and incubated at 65°C for 10 min. The mixture was then filtered with a filter column to remove cell debris and the supernatant was loaded to a GD column for DNA binding. The column was washed and the bound DNA was eluted with 50  $\mu\text{L}$  of elution buffer. To improve the lysis condition, the following modified protocols were carried out before the filtration step:

### 2.2.1 Glass bead and liquid nitrogen method (GL)

Fifty milligrams of a fresh sample was ground in liquid nitrogen with 100 - 150 mg sterile glass beads, size  $\leq 106 \mu\text{m}$  (Sigma). Then, 400  $\mu\text{L}$  of lysis buffer and 5  $\mu\text{L}$  of RNase A were added and the sample was transferred to a 1.5 mL microcentrifuge tube, mixed on a vortex mixer for 5 sec and then incubated at 65°C for 10 min and 2 h.

### 2.2.2 NaCl and glass bead method (NG)

The cells (50 mg) were washed 3 times with 300  $\mu\text{L}$  of 3 M NaCl and centrifuged at 6,000 rpm for 10 min. The supernatant was discarded. The cells were ground with 100 - 150 mg sterile glass beads, with 400  $\mu\text{L}$  of lysis buffer and 5  $\mu\text{L}$  of RNase A in a 1.5 mL microcentrifuge tube, mixed on a vortex mixer for 5 sec and incubated at 65°C for 10 min and 2 h.

### 2.2.3 Freeze thaw method (FT)

The cells (50 mg) were mixed with 400  $\mu\text{L}$  of lysis buffer and 5  $\mu\text{L}$  of RNase A in 2 mL cryogenic vial (Thermo Fisher Scientific). The vial was frozen in liquid nitrogen and thawed at 37°C in a water bath three times. The cell suspension was further transferred into a 1.5 mL microcentrifuge tube and incubated at 65°C for 10 min and 2 h.

### 2.2.4 Glass bead and sonication method (GS).

The cells (50 mg) were mixed with 400  $\mu\text{L}$  of lysis buffer, 5  $\mu\text{L}$  of RNase A and 100 - 150 mg sterile glass beads. The mixture was ultra-sonicated for 1 min at 6 kHz (Sonic Vibra cell™) under chilled conditions. The mixture was then incubated at 65°C for 10 min and 2 h.

### 2.2.5 Liquid nitrogen and polyvinylpolypyrrolidone method (LP)

The cells (50 mg) were crushed in liquid nitrogen, and then 400  $\mu\text{L}$  of lysis buffer and 5  $\mu\text{L}$  of RNase A were added. The suspension was transferred into 1.5 mL microcentrifuge tube and 5 mg of polyvinylpolypyrrolidone (PVPP) [12] was added and it was then mixed on a vortex mixer for 5 sec and incubated at 65°C for 10 min and 2 h.

### 2.2.6 Glass bead and enzyme method (GE)

The cells (50 mg) were crushed with 100 - 150 mg sterile glass beads, and 400  $\mu\text{L}$  of lysis buffer (GP1), 5  $\mu\text{L}$  of RNase A and 50  $\mu\text{L}$  of 5 mg.  $\text{mL}^{-1}$  of lysozymes were added and the mixture was incubated at 37°C for 30 min [10]. The mixture was then further incubated at 65°C for 10 min and 2 h.

## 2.3 DNA Quality and Quantity Analysis

The amplifications were performed in a MyCycler™ Thermal Cycler (Bio-Rad Laboratories, Irvine, CA, USA). The 16S rRNA gene with an 16S-23S intergenetic segment was amplified using 0.1  $\mu\text{M}$  of primers 16S27F (AGA GTT TGA TCC TGG CTC AG) [15] and 0.1  $\mu\text{M}$  of 23S30R (CTT CGC CTC TGT GTG CCT AGG) [16]. Two microliters of genomic DNA was added to the PCR reactions containing 10  $\mu\text{L}$  RedDye PCR master mix (Genei™, Merck) and water up to a final volume of 20  $\mu\text{L}$ . The PCR conditions suggested by Zapomílová *et al.* [17] was as follows: one cycle of 5 min at 94°C; 10 cycles of 45 sec at 94°C, 45 sec at 57°C, and 2 min at 72°C; 25 cycles of 45 sec at 94°C, 45 sec at 54°C, and 2 min at 72°C; and a final elongation step of 7 min at 72°C. The size of the DNA

was analyzed on 1% (w/v) agarose gel electrophoresis in 1X TAE buffer, using 1 kb DNA ladder (Fermentas) as a molecular weight marker and electrophoresed at 120 V for 35 min. The gel was stained with  $0.5 \mu\text{g} \cdot \text{mL}^{-1}$  ethidium bromide (EtBr). The gel image was captured by the gel document (Syngene Bio imaging, USA). The concentration of the extracted DNA was determined by spectrophotometry (ND-8000 system, NanoDrop Technology, Thermo Fisher Scientific Inc.) at 260 nm using  $3 \mu\text{L}$  total genomic DNA, according to the manufacturer's instructions. The ratio of nucleic acids to proteins in the sample was evaluated by the ratio of absorbance at 260 and 280 nm ( $A_{260}/A_{280}$  ratio) [18]. Other compounds such as phenolate, thiocyanate, and polysaccharide were evaluated by the ratio of absorbance at 260 and 230 nm ( $A_{260}/A_{230}$  ratio) [11].

#### 2.4 Confirmation of Improved DNA Extraction Methods on Other Cyanobacterial Strains

Other *Nostochopsis* strains, including, *Nostochopsis* sp. CM3, *Nostochopsis* sp. NT1, *Nostochopsis* sp. MP2, *Nostochopsis* sp. TISTR8894 and other high-polysaccharide content cyanobacteria, *Nostoc commune* TISTR6290 and *Phormidium* sp. AARLC009 were chosen for the confirmation of the improved DNA extraction by liquid nitrogen and the PVPP method. The quality and quantity of the extracted genomic DNA were determined as previously described.

### RESULTS AND DISCUSSION

*Nostochopsis* sp. colony is rich in mucus (Figure 1A), which is mainly in the form of a polysaccharide sheath surrounding its filament (Figure 1B), and creates a major problem in the isolation of high quality DNA [19]. In this study, the genomic DNA

was extracted using a genomic DNA Mini Kit (plant) (Geneaid) because DNA purification and recovery steps are considered more convenient, less toxic and more environmentally friendly than phenol extraction and alcohol precipitation methods. Although, the lysis buffer of the genomic DNA Mini Kit (plant) was designed for high polysaccharide organisms, Sherwood, [8] and Lokmer, [9] successfully used the DNA extraction kit on plants to extract DNA from many species of algae and cyanobacteria. However, its conventional protocol could not extract *Nostochopsis* sp. genomic DNA effectively, due to its polysaccharide content. This was evident from the absence of a band under gel electrophoresis (Figure 2A), low DNA recovery yield (Figure 4A) and high polysaccharide impurity (Figure 4C). The low quality and quantity of the extracted genomic DNA from the kit's conventional protocol lead to unsuccessful PCR (Figure 2B). In addition, an increase of the incubation time from 10 minutes to 2 hours did not improve the lysis efficiency (Figures. 3A, 4A and 4C). Therefore, the six improvements of the lysis conditions were carried out with the conventional lysis incubation time of 10 min. It was found that all the alternative methods could improve DNA extraction, as seen from the observed genomic DNA (Figure 2A). Although the amount of extracted genomic DNA was low (Figure 4A) and defiled with polysaccharides ( $A_{260}/A_{230} < 2$ ) (Figure 4C), the amplification of targets was achieved with those genomic DNAs (Figure 2B). Consequently, an increase in incubation time from 10 min to 2 h was attempted. Morin *et al.* [11] reported that the appropriate incubation time (e.g. 3-4 hour) could reduce degradation and, ultimately, high quality DNA could be obtained. The suitable incubation periods were diverse among groups of organisms [5]. In the present study, an

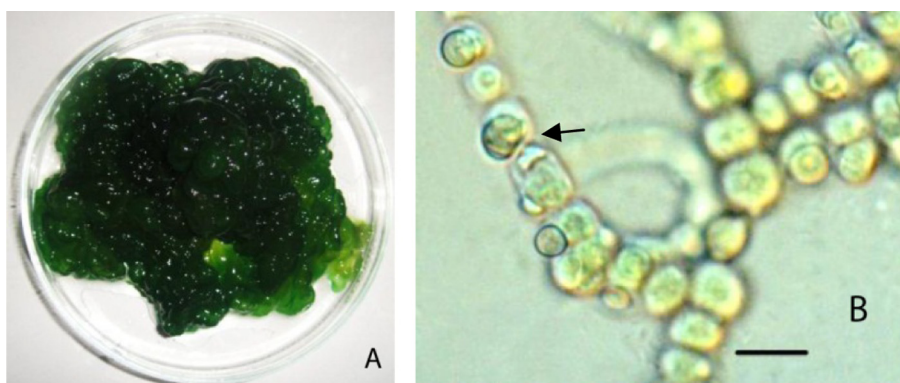
incubation time of 2 h was found to be enough for *Nostochopsis* sp. DNA extraction (Figure 3A). The longer incubation period also improved both DNA quantity and quality. The yield of extracted DNA was higher and the protein and polysaccharide impurity was lower (Figures 4A, 4B and 4C). DNA extraction with glass beads and liquid nitrogen protocol (GL) produced a moderate amount of extracted DNA (Figure 4A). However, the DNA was strongly sheared by this protocol (Figure 3B) and a high ratio of polysaccharide contamination was still present in the obtained DNA (Figure 4C). Bead-beating is used as the extraction method of nucleic acids from a wide variety of organisms for which lysis can otherwise be difficult [20]. Sample materials became more disrupted when liquid nitrogen was used in conjunction with bead mill. However, if the disruption is prolonged when the cells have been broken, the genomic DNA will be degraded [21]. Cell homogenization by glass beads, washing with NaCl (NG) and crushing it could extract a good quality genomic DNA. The sharp, dense and less shear genomic DNA band was observed (Figure 3A). Although the amount of extracted DNA was not the highest among these six methods, it was free from protein and polysaccharide contamination (Figures 4B and 4C), which could achieve a good product from gene amplification (Figure 3B). The addition of NaCl could inhibit polysaccharide and DNA co-precipitation [7]. In addition, salt or NaCl was used to remove proteins and carbohydrates, resulting in a high yield of total genomic DNA. The recommended minimum concentration of salt was 2.5 M NaCl for DNA isolation from grapevines rich in polysaccharides [22,23]. A method involving alternately freezing in liquid nitrogen and thawing at 37°C in a water bath was used to damage the cell walls and render

the cell more susceptible to further chemical and enzymatic lyses [11]. Some cyanobacterial cells such as *Spirulina* were easily lysed by freezing and thawing; however, this was difficult to achieve with *Nostochopsis* sp. The cells are surrounded with a thick sheath, which could protect from cellular glacial injury. Thus, the lightest genomic DNA band and lowest extraction yield were obtained from it (Figures 3A and 4A). The DNA from this method also showed high polysaccharide impurity (Figure 4C). Mixing *Nostochopsis* sp. cells and glass beads after sonication (GS) showed shear genomic DNA. Although this method could extract higher amounts of genomic DNA with low protein and polysaccharide impurity (Figures 4A, 4B and 4C), a lighter PCR band was observed. The addition of PVPP after the specimen was crushed with liquid nitrogen (LP) provided a dense, sharp DNA band and fewer shears (Figure 3A). In addition, this protocol provided the highest DNA extraction yield with a minimal amount of protein and polysaccharide contamination (Figures 4A, 4B and 4C). Many chemicals have been applied when dealing with high polysaccharide containing plants, such as cetyltrimethylammonium bromide (CTAB) or PVPP. Moreover, PVPP has been used to extract genomic DNA from other polyphenol-rich plants, including cotton, sugarcane, lettuce and strawberry, as well as cyanobacteria [7,24]. Incubation with lysozymes after being homogenized with glass beads (GE) resulted in a low quantity but a high quality of genomic DNA. The band was very sharp with fewer shears and the ratios of  $A_{260}/A_{280}$  and  $A_{260}/A_{230}$  were higher than 2.00 which indicated very clean DNA. Lysozymes are responsible for breaking down the polysaccharide wall of many bacteria and thus they provide some protection against infection [25 and 26]. However, only in the GE protocol, did the yield of extracted

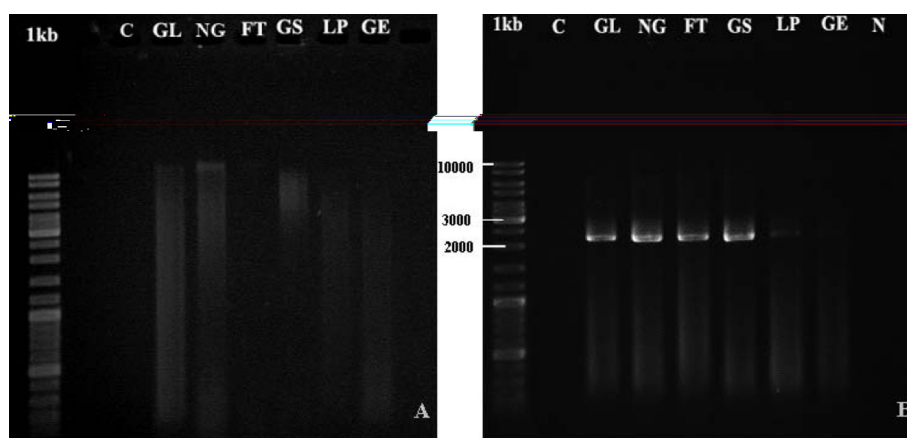


DNA decrease when the incubation period was extended. This may be the result of prolonged incubation after cell lysis. Amplification of 16S-23S rDNA was used to assure DNA quality. The PCR products were reflected from the quality and quantity of genomic DNA. It was shown that all six of the improved protocols could provide better genomic DNA than the conventional method. Michiels *et al.* [27] reported that the purification of DNA is influenced by the presence of secondary metabolites, such as polysaccharides, polyphenols, and tannins, which inhibit enzymes such as polymerases, and restrict endonucleases and ligases resulting in unsuccessful amplification. From this study, three protocols i.e., NG, GS and LP were promising for *Nostochopsis* sp. genomic DNA extraction. The pros and

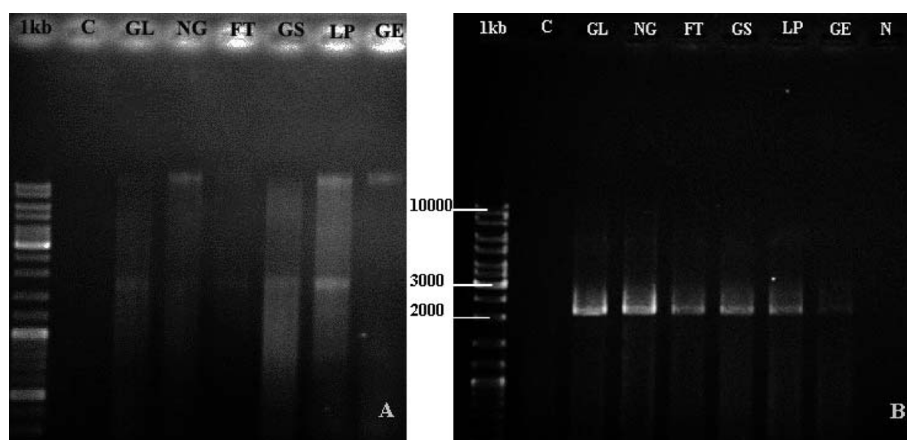
cons of each protocol are shown in Table 1. The extracted DNA showed a high amount and absorbance ratio of  $A_{260}/_{280}$  and  $A_{260}/_{230}$ , which were as high as 1.80 and 2.00. However, for convenient and efficient performance, LP method was selected for DNA extraction of other *Nostochopsis* strains and other high polysaccharide containing cyanobacteria. In which case, the cyanobacterial genomic DNA were successfully extracted (Figure 5A), but the amount varied among strains depending on the polysaccharide content (Figure 6A). In addition, the extracted DNA samples were highly free from proteins and polysaccharides and this could be a suitable template for gene amplification. (Figures 5B, 6B and 6C).



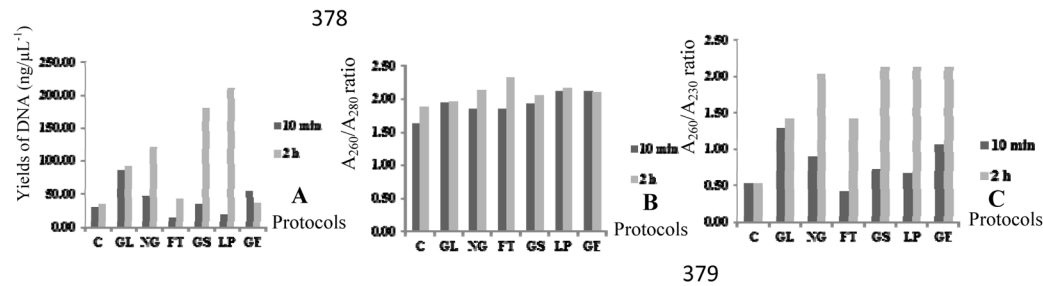
**Figure 1. A:** Mucilaginous balls of *Nostochopsis* sp. NT2 colony **B:** Filament of cultivated *Nostochopsis* sp. under light microscope showing polysaccharide sheath surrounding the cells (arrow). Scale bar = 20  $\mu$ m.



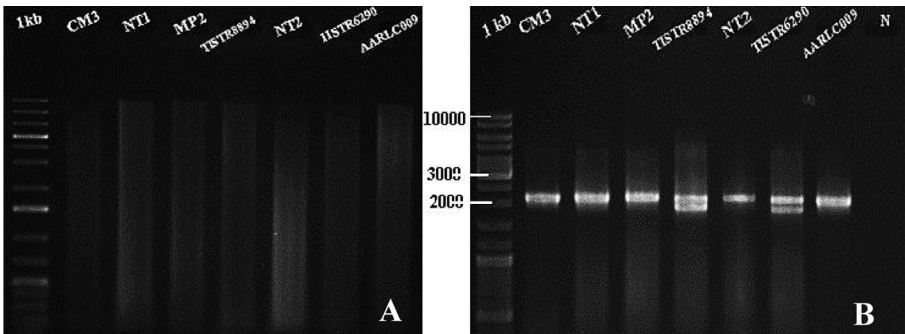
**Figure 2. A:** Genomic DNA from *Nostochopsis* sp. NT2 **B:** PCR amplification for 16S-23S rDNA gene which DNA extracted by: C; conventional protocol of genomic DNA Mini Kit (plant), GL; glass bead and liquid nitrogen method, NG; NaCl and glass bead method, FT; freeze-thaw method, GS; glass bead and sonication method, LP; liquid nitrogen and polyvinylpolypyrrolidone method (LP), GE; glass bead and enzyme method, N; PCR negative control (genomic DNA absent). Incubation time was 10 min.



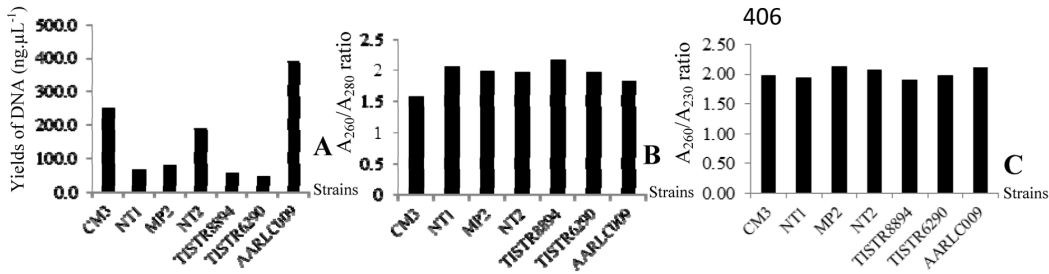
**Figure3. A:** Genomic DNA from *Nostochopsis* sp. NT2 **B:** PCR amplification for 16S-23 S rDNA gene which DNA extracted by: C; conventional protocol of genomic DNA Mini Kit (plant), GL; glass bead and liquid nitrogen method, NG; NaCl and glass bead method, FT; freeze-thaw method, GS; glass bead and sonication method, LP; liquid nitrogen and polyvinylpolypyrrolidone method (LP), GE; glass bead and enzyme method, N; PCR negative control (genomic DNA absent). Incubation time was 2 h.



**Figure 4.** A: Concentration of extracted DNA (ng.μL<sup>-1</sup>) B: Absorbance ratio 260/280 nm, C: Absorbance ratio 260/230 nm.



**Figure 5.** A: Genomic DNA of *Nostochopsis* spp. strains and other high polysaccharide content cyanobacteria B: PCR amplification for 16S-23S rDNA gene. *Nostochopsis* strains; *Nostochopsis* sp. CM3, *Nostochopsis* sp. NT1 *Nostochopsis* sp. MP2, *Nostochopsis* sp. NT2, *Nostochopsis* sp. TISTR8894, *Nostoc commune* TISTR6290 and *Phormidium* sp. AARLC009.



**Figure 6.** A: Concentration of DNA of *Nostochopsis* strains (ng.μL<sup>-1</sup>) B: Absorbance ratio 260/280 nm, C: Absorbance ratio 260/230 nm, *Nostochopsis* strains; *Nostochopsis* sp. CM3, *Nostochopsis* sp. NT1 *Nostochopsis* sp. MP2, *Nostochopsis* sp. NT2, *Nostochopsis* sp. TISTR8894, *Nostoc commune* TISTR6290 and *Phormidium* sp. AARLC009.



**Table 1.** The pros and cons of each extraction protocol.

Methods	Pros	Cons
GL	<ul style="list-style-type: none"> <li>- Moderate yield of genomic DNA</li> <li>- Extracted DNA is clear from proteins</li> <li>- DNA quality good to be template for PCR</li> </ul>	<ul style="list-style-type: none"> <li>- Genomic DNA strong shear</li> <li>- Extracted DNA is contaminated with polysaccharide</li> </ul>
NG	<ul style="list-style-type: none"> <li>- Moderate yield genomic DNA</li> <li>- Bands of genomic DNA and PCR are sharp</li> <li>- Cheap and convenient</li> <li>- Extracted DNA is clear from proteins and polysaccharides</li> </ul>	<ul style="list-style-type: none"> <li>- Low yield of genomic DNA</li> </ul>
FT	<ul style="list-style-type: none"> <li>- DNA quality good to be template for PCR</li> <li>- Extracted DNA is clear from proteins</li> </ul>	<ul style="list-style-type: none"> <li>- Low yield of genomic DNA</li> <li>- Extracted DNA is contaminated with polysaccharide</li> </ul>
GS	<ul style="list-style-type: none"> <li>- High yield of genomic DNA</li> <li>- Extracted DNA is clear from proteins and polysaccharides</li> <li>- DNA quality good to be template for PCR</li> </ul>	<ul style="list-style-type: none"> <li>- Genomic DNA strong shear</li> <li>- Require specific equipment such as sonicator</li> </ul>
LP	<ul style="list-style-type: none"> <li>- The highest yield of genomic DNA</li> <li>- Sharp band of genomic DNA and PCR</li> <li>- Extracted DNA was clear from proteins and polysaccharides</li> <li>- DNA quality good to be template for PCR</li> </ul>	<ul style="list-style-type: none"> <li>- Genomic DNA slightly shear</li> <li>- Require specific reagent, such as PVPP and liquid nitrogen</li> </ul>
GE	<ul style="list-style-type: none"> <li>- Very sharp band of genomic DNA and less shear</li> <li>- Extracted DNA is clear from proteins and polysaccharides</li> </ul>	<ul style="list-style-type: none"> <li>- Very low yield of genomic DNA</li> <li>- Require specific reagent, such as lysozyme</li> <li>- More expensive than other methods</li> </ul>

## CONCLUSION

The DNA extraction protocols for *Nostochopsis* spp. were compared. These methods used cheap, convenient and available chemicals and agents, such as NaCl, glass beads, a sonicator, liquid nitrogen and PVPP. Each method has its own pros and cons, which must be considered according to the availability in each laboratory. In addition, these lysis protocols should be applied with other DNA purification and recovery methods, such as phenol extraction and alcohol precipitation. Moreover, these

improved methods could be used for other cyanobacteria, such as *Nostoc* and *Phormidium* and other groups of algae.

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