CHAPTER 4

IMPROVEMENT OF DNA EXTRACTION PROTOCOLS

FOR *Nostochopsis* spp.

4.1 Introduction

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 food and traditional medicine (Thiamdao *et al*., 2011), Local tribes of India use it as a dietary supplement (Pandey and Pandey, 2008a). Information on this cyanobacterium is known in many countries as a cosmopolitan species (Anagnostidis and Komárek, 1988) but a few investigations on its genotypic information have been reported. However, the study on its genotype was rather difficult because isolation of genomic DNA from filamentous cyanobacteria often poses problems, due to its branching patterns and additional surface structures such as mucilaginous sheath, S-layer, pili, slime, capsule etc. (Singh *et al*., 2012). In addition, it has high content of polysaccharide up to 49% w/w (Thiamdao *et al*., 2011 and Motham *et al*., 2012b). The polysaccharide, polyphenols and other secondary metabolites make it very difficult to isolate satisfactory quality of DNA. These compounds bind tightly to nucleic acids during the isolation of DNA and interfere with subsequent reactions (Souza *et al*., 2012). Most genetic diversity studies

use molecular tools, which are mainly based on polymerase chain reaction (PCR) that requires good quality DNA. Moreover, reports on the difficulties in isolating acceptable quality DNA is common in high polysaccharide plants and many commercial companies produce genomic DNA extraction kit for these plants. They are more convenient, easier and less toxic than chemical extraction method. Although, the extraction by those DNA extraction kits for plants was successful with many mucilaginous algae (Sherwood, 2007 and Lokmer, 2007). However, it did not work with mucilaginous cyanobacteria, especially, *Nostochopsis*. The lysis buffer and lysis condition in those kits are not sufficient to obtain good quality and high quantity of DNA from these cyanobacteria. Many plant and cyanobacterial lysis methods have been developed (Feurer *et al*., 2004; Angeles *et al*., 2005; Lokmer, 2007; Yilmaz *et al*., 2009; Morin *et al*., 2010 and Singh *et al*., 2012). However, comparison of 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.

4.2. Materials and Methods

4.2.1 Cyanobacterial strains

Nostochopsis NT1 and *Nostochopsis* NT2 were collected from Nan River, Nan Province. *Nostochopsis* CM3 was collected from a glass house of Queen Sirikit Botanical Garden, Chiang Mai. *Nostochopsis* MP2 was collected from Khong Muang Canal, Mae Hong Son. *Nostochopsis* TISTR8894 and *Nostoc commune* TISTR6290

were obtained from Thailand Institute of Scientific and Technological Research (TISTR). *Phormidium* sp. AARLC009 was from the stock culture of Applied Algal Research Laboratory (AARL), Chiang Mai University. All the cultures were maintained in liquid nitrogen-free BG-11 medium (Rippka *et al*., 1979) at 24 ºC under continuous cool-white fluorescent lamp with light intensity 8.76 μmole.m^{-2}.s^{-1}. Twenty days old samples were used in each experiment.

4.2.2 DNA extraction

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

4.2.2.1 Glass bead and liquid nitrogen method (GL)

Fifty milligrams of fresh sample was ground in liquid nitrogen with 100 – 150 mg sterile glass beads, size ≤ 106 μm (Sigma). Then, 400 μL of lysis buffer and 5 μL of RNase A were added and the sample was transferred to a 1.5 mL microcentrifuge tube, mixed on vortex mixer for 5 sec and incubated at 65 ºC for 10 min and 2 h.

4.2.2.2 NaCl and glass bead method (NG)

The cells (50 mg) were washed 3 times with 300 μL 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, added with 400 μL of lysis buffer and

5 µL of RNase A in a 1.5 mL microcentrifuge tube, mixed on a vortex mixer for 5 sec and incubated at 65ºC for 10 min and 2 h.

4.2.2.3 Freeze thaw method (FT)

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

4.2.2.4 Glass bead and sonication method (GS)

The cells (50 mg) were mixed with 400 µL of lysis buffer, 5 µL of RNase A and 100 – 150 mg sterile glass beads. The mixture was ultra-sonicated for 1 min at 6 kHz (Sonic Vibra cellTM) under chilled condition. The mixture was then incubated at 65 ºC for 10 min and 2 h.

4.2.2.5 Liquid nitrogen and polyvinylpolypyrrolidone method (LP)

The cells (50 mg) were crushed in liquid nitrogen and, 400 µL of lysis buffer and 5 µL of RNase A were added. The suspension was transferred into 1.5 mL microcentifuge tube and 5 mg of polyvinylpolypyrrolidone (PVPP) (Morin *et al*., 2010) was added then mixed on a vortex mixer for 5 sec and incubated at 65 ºC for 10 min and 2 h.

4.2.2.6 Glass bead and enzyme method (GE)

The cells (50 mg) were crushed with 100 – 150 mg sterile glass beads, and 400 µL of lysis buffer (GP1), 5 µL of RNase A and 50 µL of 5 mg. mL^{-1} of lysozyme were

added and incubated at 37 ºC for 30 min (Angeles *et al*., 2005) The mixture was further incubated at 65 ºC for 10 min and 2 h.

4.2.3 DNA quality and quantity analysis

The amplifications were performed in a MyCyclerTM Thermal Cycler (Bio-Rad Laboratories, Irvine, CA, USA). The 16S rRNA gene with the 16S-23S intergenetic segment was amplified using 0.1 μM of primers 16S27F (AGA GTT TGA TCC TGG CTC AG) (Taton *et al*., 2003) and 0.1 μM of 23S30R (CTT CGC CTC TGT GTG CCT AGG) (Wilmotte *et al*., 1993). Two microliter of genomic DNA was added to PCR reactions containing 10 μL Red Dye PCR master mix (Genei^{TM}, Merck) and water up to the final volume 20 μL. The PCR condition suggested by Zapomělová *et al*. (2011) was as follows: one cycle of 5 min at 94 ºC; 10 cycles of 45 sec at 94 ºC, 45 sec at 57 ºC, and 2 min at 72 ºC; 25 cycles of 45 sec at 94 ºC, 45 sec at 54 ºC, and 2 min at 72 ºC; and a final elongation step of 7 min at 72 ºC. The size of DNA was analyzed on 1% (w/v) agarose gel electrophoresis in 1X TAE buffer, using 1 kb DNA ladder (Fermentas) as molecular weight marker and electrophoresed at 120 V for 35 min. The gel was stained with 0.5 μg.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 μL total genomic DNA, according to manufacturers instruction. 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) (Sambrook *et al*., 2001). Other compounds such as phenolate, thiocyanate, and polysaccharide were evaluated by the ratio of absorbance at 260 and 230 nm ($A_{260}/_{230}$ ratio) (Morin *et al*., 2010).

4.2.4 Confirmation of improved DNA extraction methods on other cyanobacterial strains

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

4.3 Results and Discussion

Nostochopsis colony is rich in mucus (Figure 4.1A) which is mainly in the form of polysaccharide sheath surrounding its filament (Figure 4.1B) and create a major problem in the isolation of high quality DNA (Odukoya *et al*., 2007). In this study, the genomic DNA was extracted by using genomic DNA Mini Kit (plant) (Geneaid) because DNA purification and recovery steps are more convenient, less toxic and more environmentally friendly than phenol extraction and alcohol precipitation.

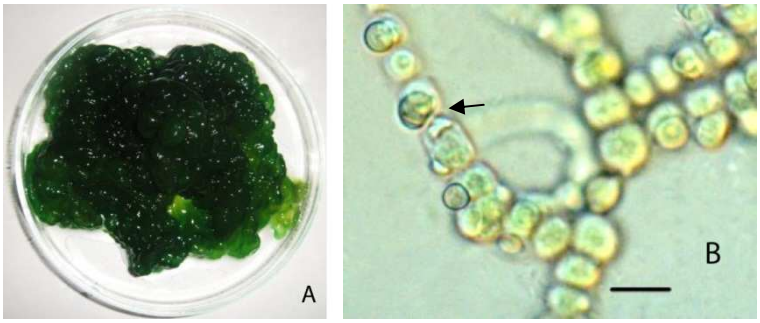

Figure 4. 1 A) Mucilaginous balls of *Nostochopsis* NT2 colony **B)** Filament of cultivated *Nostochopsis* under light microscope showing polysaccharide sheath surrounding the cells (arrow). Scale bar = 20 μm

Although, the lysis buffer of the genomic DNA Mini Kit (plant) was designed for high polysaccharide organisms Sherwood, (2007) and Lokmer, (2007) successfully used DNA extraction kit for plant to extract DNA from many species of algae and cyanobacteria, However, its conventional protocol could not extract *Nostochopsis* genomic DNA effectively, due to its polysaccharide content. It was evident from the absence of band under gel electrophoresis as can be seen in Figure 4.2A, low DNA recovery yield (Figure 4.4A) and high polysaccharide impurity (Figure 4.4C). The low quality and quantity of extracted genomic DNA from the kit conventional protocol lead to unsuccessful PCR (Figure 4.2B). In addition, increasing of incubation time from 10 minutes to 2 hours did not improve the lysis efficiency (Figures 4.3A, 4.4A and 4.4C). Therefore, the six improvements of lysis condition 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 4.2A). Although the amount of extracted genomic DNA was low (Figure 4.4A) and defiled with polysaccharide ($A_{260}/A_{230} < 2$) (Figure 4.4C), the amplification of targets was achieved with those genomic DNAs (Figure 4.2B). Consequently, an increase in incubation time from 10 min to 2 h was attempted. Morin *et al*. (2010) reported that the appropriate incubation time (e.g. 3-4 h) could reduce degradation and high quality DNA could be obtained. The suitable incubation periods were diverse among groups of organisms (Singh *et al*., 2012).

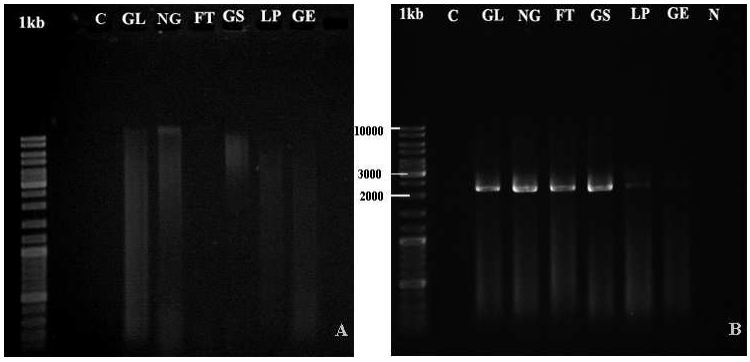

Figure 4.2 A) Genomic DNA from *Nostochopsis* NT2 **B)** PCR amplification for 16S-23S rDNA gene which DNA extracted by: **C**: conventional protocol of genomic DNA Mini Kit (plant), **GL**: glass bead and liquid nitrogen method, **NG**: NaCl and glass bead method, **FT**: freeze- thaw method, **GS**: glass bead and sonication method, **LP**: liquid nitrogen and polyvinylpolypyrrolidone method, **GE**: glass bead and enzyme method**. N:** PCR negative control (genomic DNA absent). Incubation time was 10 min.

In the present study, the incubation time of 2 h was enough for *Nostochopsis* DNA extraction (Figure 4.3A). 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 4.4A, 4.4B and 4.4C). DNA extraction with glass bead and liquid nitrogen protocol (GL) showed moderate amount of extracted DNA (Figure 4.4A). However, the DNA was strongly sheared by this protocol (Figure 4.3B) and high ratio of polysaccharide contamination was still present in the obtained DNA (Figure 4.4C). Bead-beating is the extraction of nucleic acids from a wide variety of organisms for which lysis can be otherwise difficult such as plant (Roberts, 2007). Sample materials were more disrupted by using liquid nitrogen in conjunction with bead mill. However, if the disruption is prolonged when the cells have been broken, the genomic DNA will be degraded (Roche, 2002). Cell homogenization by glass beads, washing with NaCl (NG) and crushing it could

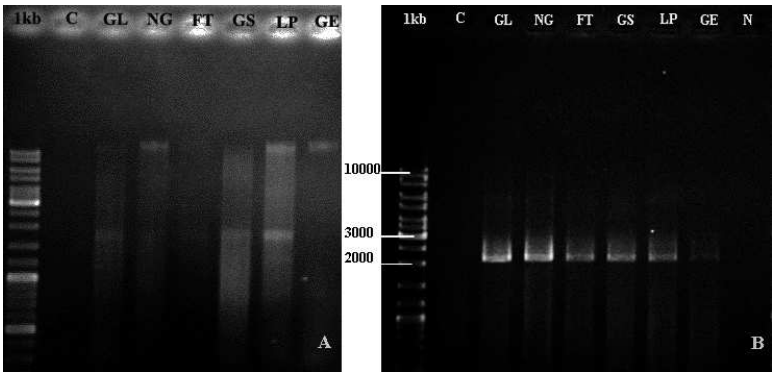

Figure 4.3 A) Genomic DNA from *Nostochopsis* NT2 **B)** PCR amplification for 16S-23S rDNA gene which DNA extracted by **C:** conventional protocol of genomic DNA Mini Kit (plant), **GL**: glass bead and liquid nitrogen method, **NG**: NaCl and glass bead method, **FT**: freeze-thaw method, **GS**: glass bead and sonication method, **LP**: liquid nitrogen and polyvinylpolypyrrolidone method, **GE**: glass bead and enzyme method**. N:** PCR negative control (genomic DNA absent). Incubation time was 2 h.

extract a good quality genomic DNA. The sharp, dense and less shear genomic DNA band was observed (Figure 4.3A). Although the amount of extracted DNA was not the highest among these six methods, it was free from protein and polysaccharide contamination (Figures 4.4B and 4.4C), which could achieve a good product from gene amplification (Figure 4.3B). Addition of NaCl could inhibit polysaccharide and DNA co-precipitation (Souza *et al*., 2012). In addition, salt or NaCl was used to remove proteins and carbohydrates, resulting in 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 (Lodhi *et al*., 1994 and Fleischmann *et al*., 2009). Alternate freezing in liquid nitrogen and thawing at 37 ºC in a water bath, were used to damage the cell walls and render the cell more susceptible to further chemical and enzymatic lyses (Morin *et al*., 2010). Some cyanobacterial cells such as *Spirulina* were easily lyzed by freezing and thawing; however, it was difficult for *Nostochopsis*.

The cells are surrounded with thick sheath which could protect cellular glacial injury. Thus, the lightest genomic DNA band and lowest extraction yield were obtained (Figures 4.2A and 4.3A). The DNA from this method also showed high polysaccharide impurity (Figure 4.4C). Mixing *Nostochopsis* cells and glass beads after sonication (GS) showed shear genomic DNA. Although this method could extract higher amount of genomic DNA with low protein and polysaccharide impurity (Figures 4.4A, 4.4B and 4.4C), lighter PCR band was observed. Addition of PVPP after being crushed with liquid nitrogen (LP) provided dense, sharp DNA band and fewer shears (Figure 4.3A).

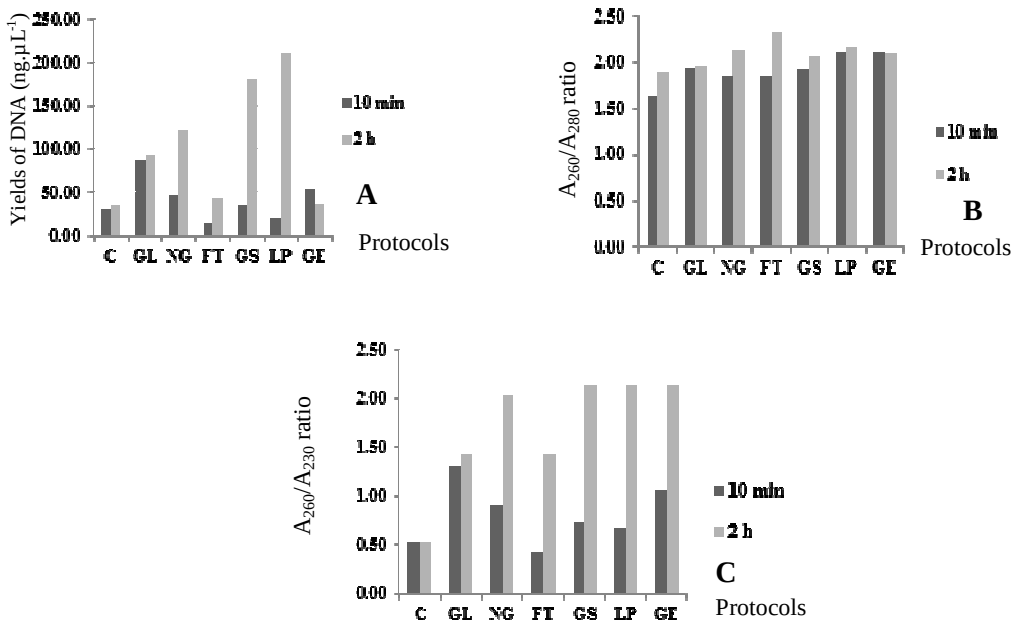

Figure 4.4 A) Concentration of extracted DNA (ng.µL^{-1}), **B)** Absorbance ratio 260/280 nm, **C:** Absorbance ratio 260/230 nm.

In addition, this protocol provided the highest DNA extraction yield with small amount of protein and polysaccharide contamination (Figures. 4.4A, 4.4B and 4.4C). Many chemicals were applied for high polysaccharide containing plant 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 (Aljanabi *et al*., 1999 and Souza *et al*., 2012). Incubation with lysozyme after being homogenized with glass beads (GE) gave low quantity but high quality 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. Lysozyme is responsible for breaking down the polysaccharide wall of many bacteria and thus it provides some protection against infection (Smalla *et al*., 1993 and Guobin *et al*., 2008). However, only in GE protocol, the yield of extracted DNA decreased when incubation period was longer. This may be the result of prolong 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 improved protocols could provide better genomic DNA than the conventional method. Michiels *et al*. (2003) reported that purification of DNA is influenced by the presence of secondary metabolites such as polysaccharide, polyphenol, and tannin which inhibit the enzymes such as polymerases, restriction endonucleases and ligases resulting in an unsuccessful amplification.

From this study, three protocols *i.e*., NG, GS and LP were promising for *Nostochopsis* genomic DNA extraction. The pros and cons of each protocol were shown in Table 4.1. The extracted DNA showed high amount and absorbance ratio of $A_{260}/_{280}$ and $A_{260}/_{230}$ 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.

Those cyanobacterial genomic DNA were successfully extracted as can be seen in Figure 4.5A but the yield varies among strains, depending on the polysaccharide content (Figure 4.6A).

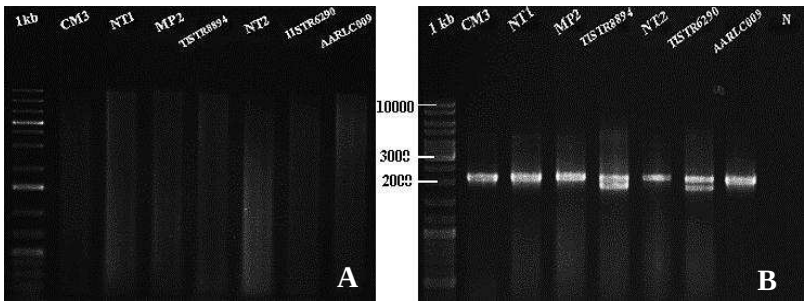

Figure 4.5 A) Genomic DNA of *Nostochopsis* and other high polysaccharide content cyanobacteria, **B**) PCR amplification for 16S-23S rDNA gene. *Nostochopsis*; *Nostochopsis* CM3,NT1 MP2, NT2, *Nostochopsis* sp. TISTR8894, *Nostoc commune* TISTR6290 and *Phormidium* sp. AARLC009

In addition, the extracted DNAs were free from protein and polysaccharide and could be a suitable template for gene amplification. (Figures 4.5B, 4.6B and 4.6C).

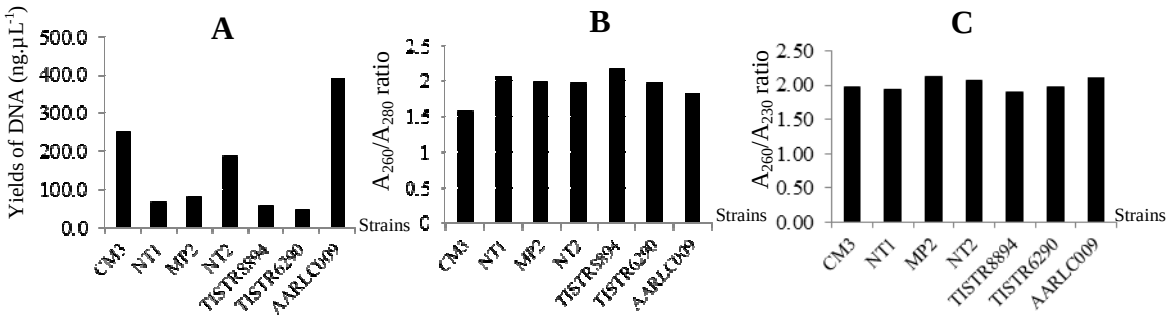

Figure 4.6 **A**) Concentration of DNA of *Nostochopsis* (ng.µl^{-1}), **B)** Absorbance ratio 260/280 nm, **C)** Absorbance ratio 260/230 nm, *Nostochopsis*; *Nostochopsis* CM3, NT1, MP2, NT2, *Nostochopsis* sp. TISTR8894, *Nostoc commune* TISTR6290 and *Phormidium* sp. AARLC009

Table 4.1 The pros and cons of each extraction protocol

Methods	Pros	Cons
GL	- Moderate yield of genomic DNA - Extracted DNA is clear from proteins - DNA quality good to be template for PCR	- Genomic DNA strong shear - Extracted DNA is contaminated with polysaccharide
NG	- Moderate yield genomic DNA - Bands of genomic DNA and PCR are sharp - Cheap and convenient - Extracted DNA is clear from proteins and polysaccharides	- Low yield of genomic DNA
FT	- DNA quality good to be template for PCR - Extracted DNA is clear from proteins	- Low yield of genomic DNA - Extracted DNA is contaminated with polysaccharide
GS	- High yield of genomic DNA - Extracted DNA is clear from proteins and polysaccharides - DNA quality good to be template for PCR	- Genomic DNA strong shear - Require specific equipment such as sonicator
LP	- The highest yield of genomic DNA - Sharp band of genomic DNA and PCR - Extracted DNA was clear from proteins and polysaccharides - DNA quality good to be template for PCR	- Genomic DNA slightly shear - Require specific reagent such as PVPP and liquid nitrogen
GE	- Very sharp band of genomic DNA and less shear - Extracted DNA is clear from proteins and polysaccharides	- Very low yield of genomic DNA - Require specific reagent such as lysozyme - More expensive than other methods