A novel RNA extraction method for cyanobacteria

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A NOVEL RNA EXTRACTION METHOD FOR CYANOBACTERIA

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DEDICATION

To my loving parents,

Su’e Liu and Chunhua Zhang,

and my special one Wenna Zhao,

who always stand behind me, support me and motivate me.
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LIST OF ABBREVIATIONS

BCP: 1-bromo-3-chloropropane

CDN A: complementary deoxyribonucleic acid

CDW: cell dry weight

CTAB: cetyltrimethylammonium bromide

DEPC: diethyl pyrocarbonate;

DNA: deoxyribonucleic acid

EtOH: ethanol

HIP: highly iterated palindrome

mRNA: messenger RNA

PHA: polyhydroxyalkanoates

RNA: ribonucleic acid

RNase: RNA ribonuclease

rRNA: ribosomal RNA

RT: reverse transcription

RT-PCR: reverse transcription polymerase chain reaction
ABSTRACT

Research on production of polyhydroxyalkanoates (PHA) by microorganisms has captured attention over the last two decades, since PHA have similar properties as petroleum-based thermoplastics, yet are close to carbon neutral and made from renewable sources. Cyanobacteria are considered to be good PHA producers because of their simple nutrient requirements (mainly water, sunlight and CO₂) and fast cell growth. However, knowledge of metabolisms behind PHA production by cyanobacteria is limited. Therefore, gene expression analysis of cyanobacteria is required to get a thorough understanding at the molecular level, and gene expression analysis requires the extraction of high quality RNA.

Extracting high quality intact RNA from cyanobacteria is problematic due to their complicated cell wall structure and excessive polysaccharides produced by cells. Previous work on RNA extraction from cyanobacteria is either strain-specific or involves handling of hazardous chemicals like toxic chaotropic agents, saturated hot phenol or liquid nitrogen.

With this work, we developed a low-cost RNA extraction method using a simple CTAB extraction buffer. Three morphologically distinct cyanobacteria strains, *Synechocystis* sp. PCC 6803, *Plectonema* sp. strain UTEX 1541, and *Nostoc muscorum* UTEX 1037 were used to testify the validity of this new method. Two traditional extraction protocols, using the Qiagen RNeasy Mini kit and TRIzol Reagent respectively for cyanobacteria were also optimized and analyzed with the same species.

The newly developed CTAB method successfully extracted total RNA of high quality and quantity from the three selected strains, and the extracted total RNA were of sufficient
quantity and quality for RT-PCR after DNase I treatment. Compared to the two traditional extraction methods, both the purity and the yield of extracted total RNA were greatly improved when using CTAB method: yields was improved up to 13 times higher, and both the $A_{260}/A_{280}$ and $A_{260}/A_{230}$ ratio indicated less contaminations in extracted RNA. Furthermore, the experimental cost of CTAB method was significantly lowered by up to 83%, yet still easy to perform.
CHAPTER 1 – INTRODUCTION

Due to rising costs and environmental concerns associated with petroleum based products, new technologies capable of producing the same products in a more sustainable way are being sought. Advances in biotechnology over the last two decades have enabled the production of sustainable and clean energy alternatives from renewable sources [1], one of which is the employment of photosynthetic microorganisms to produce energy-rich compounds [2] such as lipids for biodiesel [3, 4] and polyhydroxyalkanoates (PHA) for bioplastics [5].

PHA are a class of polyesters that can be produced by variety of microorganisms, including most genera of bacteria. The first discovered PHA is poly[(R)-3-hydroxybutyric acid] (P[3HB]) by Lemoigne in 1925, which is the most abundant polyester in bacteria [6]. The identification of hydroxyalkanoates (HA) other than P(3HB) was not reported until 1974 [7]. PHA are accumulated as carbon- and energy-storage compounds and stored as insoluble inclusions in the cytoplasm when bacteria are in low-nutrient econiches such as the mid-ocean [8, 9]. Unlike other biopolymers i.e. extracellular polymeric substances (EPS) and bacterial melanin, PHA are originally used by bacteria as a food reserve, but they could be used industrially for a totally different function [9]. For instance, extracted PHA have similar properties as some common plastics such as polypropylene [10]. Also due to their bacterial origin, there are many microorganisms evolving the ability to degrade PHA by using secreted PHA hydrolases or PHA depolymerases, which makes PHA biodegradable. Furthermore, PHA are recyclable just like the petrochemical thermoplastics [11]. Nowadays, PHA are widely used in various purposes in the material, medical and fuel industries.
Currently, the major hurdle to commercial use of PHA is production costs, which are significantly higher than those of petroleum-based plastics [12-14]. Imperial Chemical Industries (ICI) started to market PHA-derived plastics under the trade name Biopol in 1982 [9]. However, commercial applications of PHA have been limited by their high price (approximately US $16/kg today) [15]. The patents of Biopol were purchased by Monsato in 1996 and thereafter Metabolix in 2001, but Biopol is still more than twice as costly to produce as petrochemical plastics [9].

One approach to reduce the cost is to use inexpensive carbon sources for bacteria growth and PHA synthesis. Microorganisms are able to produce PHA from various carbon sources ranging from inexpensive, complex waste effluents i.e. beet/cane molasses [16], to plant oils and their fatty acids [15, 17], alkanes [18] and unrelated carbon sources like carbohydrates i.e. gluconate, fructose, acetate, glycerol and lactate [15, 19]. Another approach currently studied is the use of economical photosynthetic organisms as PHA production hosts. It is reported that some nature PHA producers, such as *Ralstonia eutropha* can produce up to 90% of cell dry weight (CDW) as PHA granules under certain culture conditions [20, 21]. Also recombinant organisms have been engineered to maximize PHA production [22]. However, the instability of the introduced PHA synthesis genes often leads to limits on the high yield of the polymers [11, 23]. Furthermore, the amount of PHA formed in the engineered organisms is highly dependent on culture conditions such as carbon supply, fermentation temperature, etc. which dramatically increase the cost of PHA production [20, 23].

Compared to the heterotrophic fermentative platform that takes advantage of high energy substrates such as sugars, the oxygenic photosynthetic prokaryotes cyanobacteria [24] are
easily cultivated with simple nutritional demands and require only sunlight, water and CO₂ [25, 26]. To date, cyanobacteria are of great interest as food supplements [27], alternative energy to replace fossil-fuel-based energy [3, 28, 29] and agricultural fertilizer [30, 31]. It is generally thought that cyanobacteria can only produce very small amount of PHA (ranging from 1 wt% to 9 wt% of CDW [32]) under photoautotrophic growth condition compared to the production amounts of other heterotrophic bacterial strains (up to 90 wt% of CDW of *R. eutropha* [20, 21]), and when the content of PHA of CDW reaches 20 – 40 wt%, it is believed that PHA could potentially be produced at a cost of $0.20 – 0.50/kg [33]. However, the low wt% content of PHA in cyanobacteria does not necessarily indicate their incapability of PHA production due to the significant difference in the cell mass of systems [34]. In contrast, cyanobacteria are able to produce considerable amounts of PHA from a mixture of acetate ions and CO₂ (up to 55 wt% of CDW of *Synechococcus* sp. MA19 [35]), which are economical carbon sources that could drastically lower the PHA production costs [34].

However, since there is little established background knowledge in this area [3], the reason why PHA production varies when adapted to different growth condition for most cyanobacteria still remains unknown. In order to develop the potential of the employment of cyanobacteria as PHA producer, it is important to thoroughly understand different aspects of cyanobacteria at the molecular level, i.e. information about cyanobacterial physiology and metabolism. As a part of such a process, obtaining reliable gene expression data is vital [36].

Gene expression analysis applications such as real time quantitative PCR and microarray strongly depend on the quality of extracted RNA and the degree of genomic DNA contamination. Therefore, RNA extraction is the first step, and often the most critical step in
many molecular biological procedures, such as cDNA synthesis, reverse transcription, cDNA library construction, and RNA amplification. The quality of extracted RNA directly influences the results of downstream applications due to the sensitivities.

Methods for extracting high-quality intact RNA from bacteria are often problematic [37]. It is especially challenging to isolate high quality RNA from cyanobacteria [36]: most cyanobacteria possess a more complicated cell wall structure (i.e. extra surface layer) compared to other gram-negative bacteria [38, 39] and produce massive quantity of mucilaginous polysaccharides [40-43] that not only significantly hinder cellular lysis, but also interfere with most nucleic acid purification protocols [44, 45]. Cyanobacteria are also rich in endo- and exonucleases and contain photosynthetic pigments which are potential inhibitors of several enzymatic reactions, especially reverse transcription [46] and PCR [47, 48].

This work introduces a simple extraction buffer containing Cetyltrimethylammonium bromide (CTAB), which is combined with a commercially available acidic phenol: chloroform solution to disrupt the cells and release nucleic acids, followed by a selective lithium chloride precipitation to differentiate ribonucleic acid. Three cyanobacterial species from three different morphological sub-groups were selected to conduct RNA extraction using this method. Two other extraction methods using the Qiagen RNeasy Mini kit and TRIzol Reagent, respectively, were also used with the same strains to compare extraction results among the three protocols. The developed method is of low cost (less than $1 per reaction), easy to perform, and could be completed within 24 hours.
CHAPTER 2 – POLYHYDROXYALKANOATES METABOLISM

Using cyanobacteria as PHA producers is one of the most promising applications to replace petroleum-based thermoplastics. However, the knowledge of PHA metabolism of cyanobacteria is limited. It is not well known what species can produce PHA and what their natural production limitation is. Of species known as PHA producers, little is known about optimum culture condition and yield.

According to previous research, cyanobacteria that produce PHA share the same central metabolism and PHA biosynthesis mechanism with other bacteria that are considered as PHA producers [5, 26, 35, 49-51]. Therefore, understanding of PHA metabolism is equally important as RNA extraction for the application of cyanobacteria as PHA producer.

2.1 Key reaction in PHA biosynthesis

PHA biosynthesis proceeds through the action of only a few enzymes, which are specifically involved in PHA formation. The essential genes involved in PHA formation are designated as pha in alphabetic order [52]. The first pha gene isolated was from Zoogloea ramigera in 1987 [53].

Figure 1. Key reaction for PHA synthesis in cyanobacteria. PhaA: β-ketothiolase; PhaB: PHA-specific acetoacetyl-CoA reductase; PhaC: PHA synthase (or PHA polymerase).

The three-step reaction represented by Ralstonia eutropha is a typical PHA biosynthesis pathway. In R. eutropha [19], two acetyl-CoA molecules are condensed to generate
acetoacetyl-CoA by a β-ketothiolase. The product is then reduced by an NADPH-dependent reductase, producing the (R)-isomer of 3-hydroxybutyryl-CoA. PHA synthase converts the (R)-3HB-CoA into PHA monomer. The key genes involved in this acetyl-CoA dimerization pathway are shown in Figure 1.

phaA, encodes β-ketothiolase, which condenses two acetyl-CoA molecules into one acetoacetyl-CoA molecule (R. eutropha, Synechocystis sp.) [54]. Multiple β-ketothiolases may be contained in certain strains, i.e. R. eutropha, Pseudomonas putida, and Rhodopseudomonas palustris CGA009. The differences between those β-ketothiolases are their specificities, and they all act in the biosynthetic pathway to PHB synthesis [11]. For instance, in R. eutropha, there are three different β-ketothiolase designated PhbA, BktB and BktC, in which BktB and BktC condense acetyl-CoA and propionyl-CoA to form β-ketovaleryl-CoA. Reduction of β-ketovaleryl-CoA to (R)-3-hydroxyvaleryl-CoA and subsequent polymerization to form P(3HB) are catalyzed by the same enzymes involved in PHB synthesis [20]. While PhaA is still the dominant β-ketothiolase, BktB or BktC can complement PhaA in PHA production [55].

phaB, encodes a PHA-specific acetoacetyl-CoA reductase, which converts the acetoacetyl-CoA into (R)-3-HB-CoA (R. eutropha, Synechocystis sp.) [54]. It is found that phaA and phaB form an operon [56]. In PHB biosynthesis pathway, the monomers for PHA polymerization are produced with the reaction catalyzed by β-ketothiolase and acetoacetyl-CoA reductase.

phaC, encodes PHA synthase (or PHA polymerase), which incorporates (R)-3-HB-CoA into PHB. The first sequenced phaC gene is from R. eutropha in 1989 [57]. PhaC is classified
into three different types with respect to their substrate specificities and primary structures [58, 59], denoted as PHA synthase I, II and III respectively. The \textit{phaC} isolated from \textit{R. eutropha} belongs to PHA synthase I. In the PHA synthase II family, at least two \textit{phaC} genes are found in \textit{R. eutropha}, \textit{R. palustris} CGA009, and most of the rRNA homology-group I pseudomonads including \textit{P. aeruginosa}, \textit{P. chlororaphis} subsp. aureofaciens, \textit{P. corrugate}, \textit{P. extremaustralis}, \textit{P. fluorescens}, \textit{P. oleovorans}, \textit{P. pseudoalcaligenes}, \textit{P. putida}, \textit{P. resinovorans}, and \textit{P. sp. USM4-55}. Studies show that the PHA polymerases encoded by those genes are 50 – 60% identical in their primary structures and have very similar substrate specificities [60]. PHA synthase III is a two-subunit enzyme encoded by \textit{phaC} and \textit{phaE} genes in \textit{Chromatium vinosum}, \textit{Thiocystis violacea} and \textit{Synechocystis} sp. [11].

It is suggested that the PHA inclusion is a hydrophobic core of amorphous PHA that is surrounded by a phospholipid monolayer membrane consisting of various catabolic and non-catabolic proteins [5, 61]. Except the PHA synthase described above, the catabolic protein PHA depolymerase and phasins are discussed below.

\textit{phaP}, encodes a non-catabolic PHA-binding protein (also known as phasin, \textit{Rhodococcus ruber}, \textit{Methyllobacterium rhodesenium}, \textit{Acinetobacter}, and \textit{Aeromonas caviae}) that determines the size and production of PHA granules [62]. \textit{PhaP} is presumed to be involved in the stabilization of the amorphous hydrophobic PHA inclusion in the hydrophilic cell cytoplasm [5]. The concentration of \textit{PhaP} is shown inversely related to the size of the granule, since the overexpression of \textit{PhaP} results in many small PHB granules [63].

\textit{phaR}, encodes a repressor protein that decreases \textit{PhaP} expression in response to PHB (\textit{P. denitrificans}, and \textit{R. eutropha}). Unlike the \textit{phaP} gene, the \textit{phaR} gene is autoregulated, which
means that the transcript level of the *phaR* gene does not depend on P(3HB) accumulation [64-67].

*phaZ* encodes intracellular PHA depolymerase, which is sometimes located in between the multiple *phaC* genes described above. The transcriptional level of *PhaZ* increases sharply during production and decreases during degradation [64]. It is found that the native granules are associated with a protein inhibitor which prevents the hydrolysis of PHA by PHA depolymerase [68]. Therefore, the intercellular degradation of P(3HB) has been shown to be a very slow process [69]. Unlike the extracellular depolymerase attacking crystalline PHA, the intracellular depolymerase is suggested to be an *exo*-type hydrolase acting at the carbonyl terminus of the polymer chain [69].

In addition, a range of other activities affect PHA accumulation, including enzymes that are involved in central metabolism, global metabolic regulation, and control and maintenance of the surface of PHA granules [11].

2.2 Central metabolism pathways related to PHA formation

2.2.1 Fatty acid β-oxidation pathway

When fatty acids are the carbon source, the fatty acid β-oxidation pathway plays an important role in PHA synthesis [70]. PHA could be formed from enoyl-CoA derivatives of the fatty acid β-oxidation pathway by the overexpression of enoyl-CoA hydratase (*PhaJ*) or 3-ketoacyl-ACP (*FabG*) [65, 71-74] (Figure 2).

*phaJ* (*maoC* or *yfcX* is homologous) encodes an (R)-specific enoyl-CoA hydratase (*Aeromonas caviae*) that converts enoyl-CoA intermediates from fatty acid oxidation pathway
directly to the \((R)\)-3-hydroxyacyl-CoA precursor. \textit{PhaJ} can convert crotonyl-CoA or pentenoyl-CoA but not octenoyl-CoA [11].

The other genes involved in fatty acid \(\beta\)-oxidation pathway are described below:

\textit{fadA}, encodes \(\beta\)-ketoacyl-CoA thiolase. Without \textit{FadA}, it is more likely that the accumulated 3-ketoacyl-CoA is converted to \((R)\)-3-hydroxyacyl-CoA directly by 3-ketoacyl-ACP reductase (\textit{FabG}, discussed later) [73].

\textbf{Figure 2. PHA synthesis with an enoyl-CoA hydratase.} \textit{FadA}: \(\beta\)-ketoacyl-CoA thiolase; \textit{FadB}: a multifunctional enzyme with hydratase and dehydrogenase activities; \textit{FadD}: fatty acyl-CoA synthetase; \textit{FadE}: acyl-CoA dehydrogenase; \textit{PhaJ}: \((R)\)-specific enoyl-CoA hydratase; \textit{PhaC}: PHA synthase.

\textit{fadB}, encodes a multifunctional enzyme with hydratase and dehydrogenase activities. In the absence of \textit{FadB}, enoyl-CoA can be converted to \((R)\)-3-hydroxyacyl-CoA by epimerase and enoyl-CoA hydratase (\textit{PhaJ}) [74]. It is found that fatty acid \(\beta\)-oxidation is weakened by knocking out of \textit{fadA} and \textit{fadB} [75] which would affect the subsequent accumulation of \((R)-(3\text{HA})\)-CoA and subsequently PHA.
*fadD*, encodes fatty acyl-CoA synthetase, which is crucial to fatty acid transport to the cell [76]. Furthermore, *FadD* is considered to play the most important role in increasing the *(R)-3-hydroxyacyl-CoA* flux to mcl-PHA synthesis [74].

*fadE* (*yafH* is homologous) encodes acyl-CoA dehydrogenase, which catalyzes the dehydration of acyl-CoA to enoyl-CoA and is considered to be rate-controlling step for β-oxidation [76, 77].

**2.2.2 de novo fatty acid biosynthesis pathway**

![Diagram](image)

**Figure 3. Direct branch of de novo fatty acid biosynthesis pathway for PHA biosynthesis.**

Acc: acetyl-CoA carboxylase; *FabA*: 3-hydroxyl-ACP dehydrase; *FabB*: 3-ketoacyl-ACP synthase I; *FabD*: malonyl CoA:ACP transacylase; *FabF*, 3-ketoacyl-ACP synthase II; *FabG*, 3-ketoacyl-ACP reductase; *FabH*, 3-ketoacyl-ACP synthase III; *FabI*, enoyl-ACP reductase; *FabZ*, 3-hydroxymyristol-ACP dehydratase; *PhaG*, acyl-ACP:CoA transacylase.

Besides acetyl-CoA dimerization, a direct branch of *de novo* fatty acid biosynthesis pathway (Figure 3), taking advantage of carbohydrates can generate mcl-PHA as well [78]. Monomers for PHA are derived from the fatty acid biosynthesis pathway as *(R)-3-hydroxyacyl-ACP* intermediates, which are converted to *(R)-3-hydroxyacyl-CoA* by
acyl-ACP:CoA transacylase (*PhaG*) [11]. Genes involved in this pathway are discussed below.

*phaG*, encodes an acyl-ACP:CoA transacylase, that converts (*R*)-3-hydroxy-acyl-ACP intermediates from fatty acid biosynthesis into (*R*)-3-hydroxyacyl-CoA [79], which can be used to form PHA through *PhaC*.

*acc* encodes acetyl-CoA carboxylase, which catalyzes the irreversible carboxylation of acetyl-CoA to malonyl-CoA, the very first step in the synthesis of long-chain fatty acid. Acetyl-CoA carboxylase is a two subunit enzyme designated as *AccA* and *AccD*. The fact that there is only one-base spacing between *accA* and *fabG1* (discussed below) suggests the co-transcription of the two genes [54].

*fabA*, encodes 3-hydroxyl-ACP dehydrase (a.k.a. β-hydroxyl-ACP dehydrase). *FabA* is most active on intermediate chain length β-hydroxyl-ACPs, but it also acts on both short and long chain saturated β-hydroxyl-ACPs [80].

*fabB* encodes 3-ketoacyl-ACP synthase I (a.k.a. β-ketoacyl-ACP synthase I)/malonyl-ACP decarboxylase, which can condense malonyl-ACP and acyl-ACP to produce fatty acid intermediate 3-ketoacyl-ACP. It is shown that *fabA* and *fabB* are co-transcribed and probably forming the *fabAB* operon in *P. aeruginosa* [81].

*fabD* encodes malonyl CoA:ACP transacylase, a transacylating protein that converts malonyl CoA into malonyl-ACP [78, 82]. Over-expression of *FabD* or *FabH* (discussed below) eliminates the rate-limiting step in fatty acid synthesis and leads to the production of P(3HB) [78].
*fabF* encodes 3-ketoacyl-ACP synthase II (a.k.a. β-ketoacyl-ACP synthase II or 3-oxoacyl-ACP synthase II), another condensing enzyme that condenses malonyl-ACP and acyl-ACP to form 3-ketoacyl-ACP.

*fabG* encodes 3-ketoacyl-ACP reductase (a.k.a. β-ketoacyl-ACP reductase or 3-oxoacyl-ACP reductase), which naturally reduces 3-ketoacyl-ACP to form (R)-hydroxyacyl-ACP in fatty acid biosynthesis. A few *FabGs* also recognize 3-ketoacyl-CoA [54]. Overexpression of *FabG* could result in increased production of (R)-3-hydroxyacyl-CoA for PHA synthesis via fatty acid degradation (discussed above) [73]. *fabG1*, one of the six *fabG* paralogs encodes the PHA-specific acetoacetyl-CoA reductase (*Haloferax volcanii*) and is considered to be essential for PHA synthesis [54].

*fabH*, encodes 3-ketoacyl-ACP synthase III (a.k.a. β-ketoacyl-ACP synthase III, or 3-oxoacyl:ACP synthase III). *FabH* is a transacylating protein that converts acetyl-ACP into acetyl-CoA, which is similar to that of *phaG* gene product [78], and a similar condensing enzyme as well as *FabB* and *FabF*. Co-expression of *PhaC* and *FabH* leads to the production of P(3HB) in the presence of glucose [83]. While overproduced *FabH* may be capable of a transacylase reaction with its own product, acetoacetyl-ACP, which results in accumulation of acetoacetyl-CoA, could be converted directly into (R)-3-HB-CoA by *PhaB*, or to (R)-3-HB-ACP by *FabG* [17].

Though *FabB*, *FabF* and *FabH* all catalyze the condensation of malonyl-ACP with acyl-ACP to form acetoacetyl-ACP [17, 84], but the three have distinct roles in the pathway [80]. It is believed that *FabB* is required for unsaturated fatty acid biosynthesis, *FabF* plays an important role in temperature control that *FabF* is more active at low temperature than
high temperature [85], and FabH catalyzes the initial condensation reaction in the pathway [80].

fabI encodes enoyl-ACP reductase, which converts trans-2-enoyl-ACP to acyl-ACP for all chain lengths [80]. Inhibiting FabI with triclosan is believed to result in mcl-PHA accumulation [84].

fabZ encodes 3-hydroxymyristol-ACP dehydratase, which is another 3-hydroxyl-ACP dehydratase which functions similarly to FabA. Both FabA and FabZ dehydratases function interchangeably in the cycles of fatty acid biosynthesis [80].

2.2.3 Tricarboxylic acid cycle

![Figure 4. PHBV formation from sugars (glucose) by the methylmalonyl-CoA pathway (R. ruber). TCA cycle: Tricarboxylic Acid Cycle; AccAB: methylmalonyl-CoA carboxytransferase; Mcm: methylmalonyl-CoA mutase; SucAB: α-ketoglutarate dehydrogenase; SucCD: succinyl-CoA synthetase with two subunits.](image)

In presence of glucose, a direct link in central metabolism between the tricarboxylic acid cycle (TCA cycle) and PHA formation is shown in Figure 4 [86]. Propionyl-CoA could be formed through the methylmalonyl-CoA pathway, which originates from one of the TCA
intermediates, succinyl-CoA. Both propionyl-CoA and acetyl-CoA are converted to PHBV by the typical Pha enzymes [87].

Important genes involved in TCA cycle pathway are discussed below:

**accAB**, encodes methylmalonyl-CoA carboxytransferase that decarboxylates methylmalonyl-CoA to propionyl-CoA [86]. acc originally encodes acetyl co-enzyme A carboxylase carboxyltransferase, which has the same locale as methylmalonyl-CoA carboxytransferase [86].

**mcm**, encodes methylmalonyl-CoA mutase, which catalyzes the isomerization of methylmalonyl-CoA to succinyl-CoA [86]. Mcm is also reported to catalyze the reverse reaction, i.e. the conversion of succinyl-CoA to methylmalonyl-CoA in the formation of propionate [88]. However, the conversion of methylmalonyl-CoA to succinyl-CoA in PHA biosynthesis of *R. ruber* has been confirmed [86].

**sucAB**, encodes α-ketoglutarate dehydrogenase, which catalyzes the oxidative decarboxylation of α-ketoglutarate resulting in succinyl-CoA.

**sucCD**, encodes the two subunits of succinyl-CoA synthetase (beta subunit and alpha subunit respectively), which converts succinyl-CoA into succinate by the hydrolytic release of coenzyme A.
CHAPTER 3 – RNA EXTRACTION FROM CYANOBACTERIA

3.1 Difficulties when extracting RNA from cyanobacteria

3.1.1 Special structure of the cyanobacterial cell wall

Table 1. Summary of sub-groups of cyanobacteria

<table>
<thead>
<tr>
<th>Groups</th>
<th>Morpha</th>
<th>Major characteristics</th>
<th>Putative affects on extraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Section I</td>
<td>Unicellular</td>
<td>Simplest structure [38]</td>
<td>Similar as other gram-negative bacteria</td>
</tr>
<tr>
<td>Section II</td>
<td>Unicellular</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Section III</td>
<td>Filamentous</td>
<td>Extra surface-layer [38]</td>
<td>Hinder the cell wall lysis</td>
</tr>
<tr>
<td>Section IV</td>
<td>Filamentous</td>
<td>Extra surface-layer; three-dimensional architecture is formed by moniseriate aggregate [38]</td>
<td>Greatly hinder the cell wall lysis</td>
</tr>
<tr>
<td>Section V</td>
<td>Filamentous</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Based on the differences in structure and development, cyanobacteria are classified into five large sub-groups (Table 1) [38]. Both Section I and Section II cyanobacteria are unicellular, in which Section I cyanobacteria have the simplest structure and are the closest to the other major groups of gram-negative, unicellular bacteria (Figure 5). Cyanobacteria of Section III and Section IV have a filamentous cell structure, called a trichome [38]. During the growth of the peptidoglycan layer of filamentous cells, the transverse wall and sequentially outer membrane layer are formed. In Section III cyanobacteria, the trichome is composed solely of vegetative cells [38]. Many members of Section IV and V can produce thick-walled resting cells known as akinetes [89]. Sections IV and Section V cyanobacteria often reproduce cells by formation of hormogonia and sometimes by germination of akinetes. Beginning with the initially uniseriate structure (one cell thick), unbranched and devoid of heterocysts hormogonia are formed from the lateral branches [38], which eventually results in
a multiseriate aggregate by division in multiple planes. The three-dimensional architectures of Sections IV and V cyanobacteria are similar to those of plant tissues. Plant tissues which are considered to be much more difficult to isolate high quality RNA from than from cultured cells, because the multiseriate aggregate must be dismantled and removed first compared to disruption of a cell monolayer [90].

Figure 5. Schematic illustration of cyanobacteria cell wall structure. (Drawn by ChemBioDraw Ultra Version 12.0) S-layers are bound to the outer membrane. In cyanobacteria except Section I cyanobacteria, a mucilaginous sheath, of varying thickness, density and ultrastructure, always covers the S-layer [38, 39].

Furthermore, unlike unicellular Section I and Section II cyanobacteria that are similar to gram-negative bacteria, the vegetative trichomes of filamentous cyanobacteria are often enclosed by a tubular sheath (surface-layer, S-layer) [38, 39]. When reproduction takes places by trichome breakage within a sheath, “false branching” occurs compared to the “true” branching (i.e. dichotomous) in cyanobacteria of Section V [38]. The surface-layer is first identified in *Gloeocapsa alpicolain* in 1972 [91], and was subsequently found in most of species of unicellular *Chroococcales* and several species of filamentous *Oscillatoriales* [39]. The S-layer, which is believed to provide protection, integrity, and adhesion for the cell [92], makes cell breakage extremely challenging when performing cell homogenization.
Additionally, the peptidoglycan layer found in cyanobacteria is considerably thicker than that of most gram-negative bacteria [93], ranging from 10 nm in *Synechococcus* [94] to over 700 nm in *Oscillatoria princeps* [95].

### 3.1.2 Enriched polysaccharides in cyanobacteria

Many cyanobacteria produce massive quantities of mucilaginous polysaccharides [40-43], which not only significantly hinder cellular lysis due to the thick cell wall containing polysaccharides [39], but also interfere with most nucleic acid purification protocols due to the co-precipitation of polysaccharides with nucleic acids in low ionic strength buffer [44, 45]. The polysaccharides found in cyanobacteria are divided into three groups: storage, cell envelope and exocellular polysaccharides [40]. Cyanobacteria are capable of accumulating polysaccharides as a carbon and energy reserve for cells [96, 97]. In addition, cyanobacterial polysaccharides are found in the peptidoglycan layer [42] and in the sheath of the cell wall [40, 98]. Furthermore, cyanobacteria are reported to secrete polysaccharides during the cell growth [41], and the secreted the moderately-soluble polysaccharides can form a gelatinous layer around the cells [43, 99]. The high levels of polysaccharides as well as phenolics, lipids, and endogenous RNases yield poor quality RNA or no RNA at all in extraction procedures, since the biomolecules such as lipids, polysaccharides and polyphenolic compounds co-precipitate with RNA in a low ionic strength buffer [45], making it difficult to separate RNA from the rest of the cellular debris to get relatively clean RNA. The carry-over polysaccharides absorb mainly at 230 nm contributing a low $A_{260}/A_{230}$ ratio [100]. The contaminated RNA is not suitable for cDNA synthesis, reverse transcription PCR, *in vivo* transcription, and Northern analysis [100]. Plant tissues are believed to contain high levels of
phenol compounds, carbohydrates, polysaccharides and other unidentified compounds like cyanobacteria [101]. Several methods have been described to overcome this problem in samples rich in polysaccharides: the use of soluble polyvinylpyrrolidone (PVP) and ethanol precipitation [101]; the modified pine tree RNA isolation method [102, 103]; a modification of the acid guanidium thiocyanate-phenol-chloroform method [104]; modified TRIzol method with potassium acetate and EtOH [45]; and a bead-phenol-chloroform (BPC) method [105]. According to our knowledge, only the BPC method successfully extracts high yield total RNA from cyanobacteria, but it involves the use of hazardous chemical, i.e. saturated phenol. Furthermore, it didn’t provide any data of $A_{260}/A_{230}$ ratio, which directly indicates the level of polysaccharides contamination [105].

3.2 RNA extraction for cyanobacteria

Due to the complicated cell structure of cyanobacteria, two key factors are taken into account for extracting high quality total RNA: a sufficient homogenization method to release nucleic acids from the cell, and a proper extraction solution to separate RNA from polysaccharides during the purification step.

Because cyanobacteria are highly diverse in cell size, morphology, and cell wall composition [38, 106], various methods are used to extract RNA from different cyanobacteria including the use of the Qiagen RNeasy Mini kit for *Synechocystis* sp. [107] and *Nostoc punctiforme* (highly similar to *N. muscorum* in morphology) [108], the use of the guanidinium thiocyanate-phenol-chloroform method (commercially available as TRIzol from Invitrogen) for *N. punctiforme* [36], and the use of the acidic phenol method for *N. punctiforme* [109], *Synechocystis* sp. and *Nostoc* sp. [105]. However, all the methods
mentioned above are either species-specific or involve the use of hazardous chemicals, i.e.
toxic chaotropic agents, saturated phenol or liquid nitrogen.

3.2.1 Homogenization

<table>
<thead>
<tr>
<th>Classification</th>
<th>Methods</th>
<th>Entails</th>
<th>Pros</th>
<th>Cons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzymatic method</td>
<td>Enzyme digestion</td>
<td>Proteinase K lysosome</td>
<td>High lysis efficiency with outer layer of gram-negative bacteria</td>
<td>Prolonged lysis time when digesting complicated colony structure may result in RNA degradation</td>
</tr>
<tr>
<td>Mechanical method</td>
<td>Cryogenic grinding</td>
<td>Liquid nitrogen</td>
<td>High efficiency with disruption of plant tissue</td>
<td>Handling of liquid nitrogen; high risk of introducing exonucleases</td>
</tr>
<tr>
<td>Mechanical method</td>
<td>Beads beating</td>
<td>Zirconia/glass beads</td>
<td>High efficiency with disruption of plant tissue</td>
<td>Extra step to sterilize the beads to prevent RNase contamination</td>
</tr>
<tr>
<td>Mechanical method</td>
<td>Temperature stressing</td>
<td>Water bath/heat block</td>
<td>Easy to perform</td>
<td>May result in RNA degradation or RNA profile change</td>
</tr>
</tbody>
</table>

The idea behind homogenization is simply to increase the surface area of the biological material to afford the lysis buffer a better chance to lyse cells [90]. Various methods have been proposed to sufficiently disrupt the cells for high-quality RNA extraction and purification (Table 2), which could be classified into two main categories: Chemical methods and mechanical methods. chemical methods such as lysozyme or proteinase K digestion are normally used for nucleic acid extraction due to their efficient lysis of the outer layers of gram-negative bacteria [110]. However, for plant-tissue-like *Nostoc* genera, lysozyme digestion is not sufficient for efficient lysis due to the multi-plane three-dimensional architecture of *Nostoc* surrounded by a thin spreading film [111]. Though a prolonged incubation time can eventually lyse the cell of *Nostoc* as well as that of single-plane cyanobacteria, the long incubation time could risk the chance of exposing the vulnerable
RNA to the introducing exogenous nucleases and leading to degradation. Mechanical homogenizations used in nucleic acid isolation include physical interruption involving cryogenic grinding [108], beads beating [105], and stress caused by high temperatures [36]. Since temperature stress and cryogenic grinding could lead to possibly RNA degradation and RNA expression profile changing, beads beating is preferred for homogenization of RNA extraction. Bead beating is used when chemical lysis is inefficient or when chemical lysis yields of RNA are unacceptably low [90]. Beads collide with the cellular sample, cracking open the cell to release intercellular components [90]. The yields of both DNA and RNA from samples processed in this manner are generally much greater than through the use of chemical extraction alone [90].

3.2.2 Cetyltrimethylammonium bromide (CTAB)

Cetyltrimethylammonium bromide (CTAB) is a cationic detergent that can precipitate nucleic acids with acidic polysaccharides under low ionic strength conditions, while allowing protein and neutral polysaccharides to remain in solution [112, 113]. In contrast, in high ionic strength solution, CTAB does not precipitate with nucleic acids and instead form a complex with proteins. Therefore, CTAB is particularly useful for purification of nucleic acids from organisms that produce large quantities of polysaccharides, e.g. plants [102, 114, 115] and certain gram-negative bacteria [112, 113]. CTAB has been used widely in nucleic acid protocols for a variety of species. An extraction buffer contains 2% CTAB was used to extract high quality RNA from pine tree tissues containing a high concentration of
polysaccharides and polyphenols [102]. High quality DNA has also been isolated from wheat, peas, oats, carrots, mung beans, tobacco, *Minulus*, and various species of *Astriplex* by using a 1% CTAB extraction buffer [114]. Finally, 2% CTAB extraction buffer has been used to extract intact RNA from green microalga *Ankistrodesmus convolutus*, a species which is believed to produce appreciable amount of polysaccharides like cyanobacteria [115].

### 3.2.3 Research objectives

Based on our knowledge of cyanobacteria, when using the newly developed protocol using the extraction buffer containing 2% CTAB to extract total RNA from the selected cyanobacteria strains, we should be able to:

1. Obtain more RNA when compared to the Qiagen and TRIzol method, especially those from the filamentous cyanobacteria with three-dimensional structure, i.e. *Nostoc*;

2. Eliminate the polysaccharides contamination due to the characteristics of CTAB when utilized in nucleic acids isolation, thus a significant improvement of $A_{260}/A_{230}$ ratio should be seen from spectrophotometry;

3. Obtain intact total RNA with minor or no degradation, and suitable for sensitive downstream application such as reverse transcription.
CHAPTER 4 – MATERIALS AND METHODS

4.1 Experimental design

4.1.1 Strains

In the present work, three morphologically distinct cyanobacteria were selected for current study according to Rippka’s classification [38]. The first species selects was the Section I cyanobacteria *Synechocystis* sp. PCC 6803, the first well-sequenced photosynthetic organism, and the best-characterized cyanobacterial species. *Synechocystis* sp. often occurs in single-cell form instead of as cell aggregates (Figure 7 A), and this species has similar cell structures to other common groups of gram-negative, unicellular bacteria [116]. As a unicellular mesophilic cyanobacterium, *Synechocystis* sp. retains robust growth characteristics, and it is easy to extract RNA from it using various simple and fast RNA extraction methods [105, 107, 117]. The second species examined was the Section III cyanobacteria *Plectonema* sp. UTEX 1541 (LPP strain PCC 6402), a strain known as a non-PHA producer [32]: it is a model negative control for research on PHA metabolism of

![Microscopic photos of selected cyanobacteria strains](image)

*Figure 7. Microscopic photos of selected cyanobacteria strains.* (A) *Synechocystis* sp. PCC 6803: unicellular Section I cyanobacteria, cell occurs singly; (B) *Plectonema* sp. UTEX 1541: filamentous Section III cyanobacteria; (C) *Nostoc muscorum* UTEX 1037: filamentous Section IV cyanobacteria, multi-plane chains are formed by filaments.
cyanobacteria. *Plectonema* sp. is designated as LPP (*Lyngbya, Plectonema, and Phormidium*) group B [38, 118], whose trichomes are surrounded by consistent sheath material [38]. This sheath makes the RNA extraction from *Plectonema* more difficult than from *Synechocystis* sp.. *Plectonema* sp. is a filamentous non-heterocystous cyanobacteria that divides in only one plane [38] (Figure 7 B). To our knowledge, this is the first work on RNA extraction from *Plectonema* sp.. The third species is the Section IV cyanobacteria *Nostoc muscorum* strain UTEX 1037 whose spherical vegetative cells are not initiated adjacent to heterocysts [89] and are formed in chains [38] (Figure 7 C). Despite the immotile, ensheathed trichomes, the production of thick-walled resting cells known as akinetes are often developed during the reproduction by random trichome breakage [38, 89], forming multi-plane chains which makes *N. muscorum* more plant tissue-like. *Nostoc* is notorious for the difficulty of extracting a high yield of good quality RNA from since the cells must be dissociated before the cell walls disruptions [90]. Structural characteristics of the three cyanobacterial strains are summarized in Table 3.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Group</th>
<th>Order</th>
<th>Morpha</th>
<th>S-layer</th>
<th>Division</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Synechocystis</em> sp. PCC 6803</td>
<td>Section I</td>
<td>Chroococcales</td>
<td>Unicellular</td>
<td>Absent</td>
<td>Single plane</td>
</tr>
<tr>
<td><em>Plectonema</em> sp. UTEX 1541</td>
<td>Section III</td>
<td>Oscillatoriales</td>
<td>Filamentous</td>
<td>Present</td>
<td>Single plane</td>
</tr>
<tr>
<td><em>Nostoc muscorum</em> UTEX 1037</td>
<td>Section IV</td>
<td>Nostocales</td>
<td>Filamentous</td>
<td>Present</td>
<td>Multiple planes</td>
</tr>
</tbody>
</table>

4.1.2 Culture conditions

All selected strains were cultivated in 50-ml conical tubes with 30 ml of BG-11 medium [38] at 28°C with a light intensity of 50 μmol photons m⁻² s⁻¹. The cultures were harvested when they reached mid-logarithmic phase of growth (cell concentration of 10⁷-10⁸/ml) [117]
by concentrating to a new 15-ml conical tube, and the cells were pelleted by centrifugation and stored at -80°C until RNA was extracted.

4.1.3 Quality assessment of total RNA

Two strategies were used for assessing the quality of extracted RNA: spectrophotometry and electrophoresis [119]. From the spectrophotometric analysis, three absorbance values are usually taken into account identifying the possible contaminations caused by the presence of excess salt, contaminating protein and/or carryover organic solvents. The ratio between the absorbance at 260 nm and at 280 nm is expected to be around 2.0, while the expected 260 nm/230 nm ratio should be higher than 2.0. Any values out of the ranges mentioned above may indicate impurities existing in the RNA sample, which could possibly affect use of the RNA for downstream applications such as reverse transcription (RT), real time PCR, microarray analysis etc. Electrophoresis is able to visualize the integrity of the ribosomal RNA sub-units as well as the possible presence of low-weight RNA degradation or genomic DNA contamination. Ideally, only ribosomal RNA sub-units should be seen from electrophoresis analysis [36, 119].

4.1.4 Reference gene selection for RT-PCR

Reverse transcription (RT), the process of synthesizing DNA using messenger RNA as a template, is known to be inhibited by endonucleases, exonucleases, photosynthetic pigments and polysaccharides [46, 120], making RT-PCR a suitable test to determine if the extracted RNA is suitable for common downstream application.

Three references genes from independent pathways were selected to minimize the effects of co-regulation due to their stabilities under culture condition variations (Table 4).
Table 4. Reference genes used in RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>cpcA</td>
<td>Phycocyanin α-subunit</td>
<td>[48]</td>
</tr>
<tr>
<td>rRNA ITS</td>
<td>16S-23S ribosomal RNA internal transcribed spacer</td>
<td>[110]</td>
</tr>
<tr>
<td>nifH</td>
<td>Fe protein, a multisubunit protein of Nitrogenase</td>
<td>[129]</td>
</tr>
</tbody>
</table>

Due to the absence of the 3’ polyadenylation extension in cyanobacteria mRNA, it is difficult to remove rRNA from cyanobacteria RNA preparations [117]. A short palindrome, designated the HIP (highly iterated palindrome) element, is found abundantly throughout most cyanobacteria genes but is absent in rRNA and tRNA genes [117, 121]. RT-PCR using the HIP primer can generate a fingerprint for specific species under robust PCR condition, and the amplified cDNA products can be used for further downstream applications [117]. Due to the high abundance of HIP, unlike the other reference genes, the generation of HIP fingerprint is independent of the culture condition. Thus a HIP primer is also used to examine the quality of extracted RNA.

4.2 RNA extraction and purification

4.2.1 Homogenization

The homogenization process for all three protocols was conducted with a beads mix consisting of 0.8 g of 0.1 mm glass beads and 2.0 g of 0.5 mm glass beads (Biospec, Cat. # 11079101 and 11079105). The beads were sterilized by soaking in 1:10 dilution of bleach for 10 min, rinsing with deionized water and subsequently baking at 550°C for 1 hour. The beads mix and lysis buffer (RLT Buffer in Qiagen protocol, TRIzol Reagent in TRIzol protocol, and CTAB lysis Buffer in CTAB protocol) were pre-mixed before loading with the frozen cells. Beads beating was performed in terms of alternated vigorous vortexing and cooling on ice for 1 min each for 10 times with the respective lysis buffer.
4.2.2 CTAB protocol

1 ml of CTAB lysis Buffer (100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, 2% (w/v) CTAB, pH 8.1) was prepared and loaded with equal volume (1 ml) of phenol/chloroform (PC, pH 5.2, MP Biomedicals Cat. # 04802514) and 0.2 volume (0.2 ml) of 2-mercaptoethanol on the frozen cells. Beads mix was added before performing beads beating. The lysate was centrifuged for 10 min (2300 g, 4°C) after beads beating and subsequent 5 min incubation on ice to settle the cell debris. The aqueous phase was extracted with equal volume (1 ml) of PC and 0.2 volumes (0.2 ml) of 1-bromo-3-chloropropane (BCP, Acros Organics, Cat. # 6862500) subsequently. 0.05 volumes (50 µl) of 5 M NaCl and 2.5 volumes (2.5 ml) of 100% EtOH were then added, mixed and incubated at -20°C for at least one hour. RNA was pelleted by centrifugation for 40 min (3000 g, 4°C), and dissolved in 50 µl of TE buffer (pH 7.5). A selective precipitation was performed by adding equal volume (around 100 µl) of 4 M LiCl and incubating at 4°C overnight. The RNA pellet was obtained by a final centrifugation for 30 min (20000 g, 4°C) and subsequent washing with 70% and 80% EtOH respectively. After air drying at room temperature, the RNA pellet was dissolved in 50 µl of RNase-free water.

4.2.3 Qiagen RNeasy® mini kit

The protocol was optimized specifically for cyanobacteria according to Qiagen supplementary protocol for total RNA purification from bacteria [122] and briefly described below: 0.01 volume of 2-mercaptoethanol were pre-mixed with 1 volume of RLT Buffer to inactivate the RNase activity. The frozen cells were suspended in 700 µl of prepared RLT Buffer with the beads mix and beads beating was performed. Lysates were mixed with an equal volume of 70% EtOH (around 700 µl) and centrifuged for 10 min (3000 g, 26°C) to
settle the beads and cell debris. The supernatant was applied onto spin column, and the
flow-through was discarded after centrifugation (max, 30s). 700 µl of RW1 Buffer and two
500 µl RPE Buffer were applied subsequently to wash off the possible organic contaminants.
The RNA bound to the silica membrane was finally eluted by 50 µl of RNase-free water.

4.2.4 TRIzol reagent

The protocol was optimized for cyanobacteria according to the standard TRIzol RNA
isolation protocol provided by Molecular Research Center [123]. 2 ml of TRIzol Reagent
(Invitrogen, Cat. # 15596-08) and beads mix were loaded to resuspend the frozen cell pellet
and beads beating was performed. After centrifugation for 10 min (2300 g, 4°C), the aqueous
(upper) phase was transferred into a new tube and incubated at room temperature for 5 min.
0.2 volume (400 µl) of BCP was added and mixed by vortexing for 15s. The lysate was
incubated at room temperature for 10 min, and centrifuged for 15 min (2300 g, 4°C) for phase
separation. 500 µl of isopropanol was added to the transferred aqueous phase with 500 µl of
High Salt Precipitation Solution (1.2 M NaCl, 0.8 M sodium citrate). RNA was pelleted by
centrifugation for 8 min (3000 g, 4°C) after the mixture was incubated at room temperature
for 10 min. After washing with 70% and 80% EtOH respectively and subsequently air drying,
the RNA pellet was finally dissolved in 50 µl of RNase-free water.

4.3 Quality assessment of total RNA

RNA quality and quantity were measured by monitoring the \( \frac{A_{260}}{A_{280}} \), \( \frac{A_{260}}{A_{230}} \)
absorbance ratios and wavescan from 220 nm to 340 nm using NanoVette Beckman Coulter
Life Science UV/Vis spectrophotometer (Beckman Coulter, Germany) according to
manufacturer’s instructions. RNA integrity was visualized by electrophoresis on a 1.2%
agarose gel (Agarose Low EEO, US Biological, Cat. # A1016) in 0.5×TBE buffer. The
electrophoresis device (Thermo Scientific, Owl B1 EASYCAST Mini Gel System) was
decontaminated by soaking in 3% H₂O₂ (diluted from 30% Hydrogen Peroxide Solution,
EMD Chemical, Cat. # HX0640-5) for 10 min, rinsing with DEPC-treated water and EtOH,
and subsequently to air drying before casting the gel to prevent any possible RNA
degradation resulting from introducing RNase by handling.

4.4 RT-PCR analysis

Prior to RT, the obtained total RNA was treated with DNase I by using the TURBO
DNA-free™ Kit (Ambion, Cat. # AM1907) according to the manufacturer’s instructions to
avoid the interference of genomic DNA contamination. The DNase treatment was performed
for up to 10 μg of total RNA in a 40 μl reaction compose of 4 μl (0.1 volume) of 10×TURBO
DNase Buffer, 1 μl of TURBO DNase, and the rest nuclease-free water. After incubated at
37°C for 30 min, 4 μl of resuspended DNase Inactivation Reagent were added to the reaction
solution and incubated at room temperature for 5 min. Mixing occasionally was required
during the 5 min incubation to redisperse the DNase Inactivation Reagent, which removes the
excessive DNase enzyme and divalent cations, i.e. magnesium and calcium, that could
catalyze RNA degradation when heating. The treated RNA was obtained in the supernatant by
centrifuging at 10,000 g for 5 min at room temperature.

To perform RT, approximately 1 μg of DNase-treated total RNA was used as template
using the PrimeScript™ One-Step RT PCR Kit ver. 2 (Clontech, Cat. # RR055A) with a
gene-specific primer and the PrimeScript™ RT PCR Kit (Clontech, Cat. # RR014A) with an
HIP primer. Three sets of primers corresponding to three reference genes were picked to
perform gene-specific RT-PCR [110, 124, 125] and a highly repeated, dodecameric palindromic sequence (W2-HIPAC) [117] was picked to generate a species-specific fingerprint from different species via RT to avoid the interference of specific gene expression level under certain culture conditions. The primer sequences and their corresponding genes were shown in Table 5. Gene-specific RT-PCR was performed according to the manufacturer’s instruction, except for the use of a gene-specific annealing temperature and 35 cycles during the amplification step. The specific PCR program for One-step RT PCR and annealing temperature for the specific gene primer are shown in Figure 8 and in Table 6, respectively. For RT-PCR using the HIP primer, 1 μg (up to 8 μl) of extracted total RNA was incubated with 1 μl of HIP primer (20 μM) and 1 μl of dNTP mix (10 mM each) at 65°C for 5 min for denaturation. The 10 μl of denaturation mix were then used for a 20 μl RT reaction including 4 μl of 5× PrimeScript Buffer, 0.5 μl of RNase inhibitor, and 0.5 μl of PrimeScript RTase, which was conducted by incubation for 10 min at 30°C to obtain sufficiently long cDNA before annealing the primer, incubating at 42°C for 30 min and terminating at 95°C for 5 min. 5 μl of the RT reaction were used for PCR. 1 μl of HIP primer (20 μM), 2.5 U of Takara EX Taq HS, 2 μl of dNTP mixture (10 mM each) and 5 μl of 10x PCR Buffer II were used in a 50 μl reaction volume. The PCR was performed for 30 cycles (35 cycles for Plectonema sp.) of 94°C for 30 s, 40°C for 30 s, 72°C for 1 min. The samples were maintained at 4°C until they were analyzed on a 2% agarose gel in 0.5× TBE buffer.
Table 5. Sequences of primers designed specific for reference genes. Oligonucleotide sequences written in 5’ to 3’ orientation. Underlined sequences are those of the linked universal sequencing primers which were attached to assist in successive sequencing of PCR products.

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Name</th>
<th>Sequence</th>
<th>Tm</th>
<th>Strain</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>cpcA</td>
<td>cpcA-F2</td>
<td>ATG AAA ACC CCI CTI ACI GAA G</td>
<td>59.2°C</td>
<td><em>Synechocystis</em> sp. PCC 6803, Nostoc muscorum</td>
<td>[125]</td>
</tr>
<tr>
<td></td>
<td>cpcA-R1</td>
<td>ACC GTG GTG AGC TTT GAT GT</td>
<td>54.4°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rRNA ITS</td>
<td>16SCTS</td>
<td>CAA TTA ACC CTC ACT AAA GGT CGT AAC AAG G</td>
<td>58.8°C</td>
<td><em>Synechocystis</em> sp. PCC 6803, <em>Plectonema</em> sp., Nostoc muscorum</td>
<td>[110]</td>
</tr>
<tr>
<td></td>
<td>23SRTIS</td>
<td>CGC TCT AGA ACT AGT GGA TCC CTC TGT GTG CCT AGG TAT C</td>
<td>66.7°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>nifH</td>
<td>nifHCYANOF</td>
<td>GGA ATT CCT GYG AYC CAN ARG CNG A</td>
<td>61.9°C</td>
<td><em>Plectonema</em> sp., Nostoc muscorum</td>
<td>[129]</td>
</tr>
<tr>
<td></td>
<td>nifHCYANOR</td>
<td>CGG ATC CGD NGC CAT CAT YTC NCC</td>
<td>63.3°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complete sequence</td>
<td>W2-HIPAC</td>
<td>WGW GAT CGC CAC AC</td>
<td>-</td>
<td><em>Synechocystis</em> sp. PCC 6803, <em>Plectonema</em> sp., Nostoc muscorum</td>
<td>[117]</td>
</tr>
</tbody>
</table>
Table 6. Annealing temperatures ($T_a$) for different sets of gene-specific primers

<table>
<thead>
<tr>
<th>Strains</th>
<th>cpcA</th>
<th>rRNA ITS</th>
<th>nifH</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Synechocystis</em> sp. PCC 6803</td>
<td>53.0°C</td>
<td>54.0°C</td>
<td>-</td>
</tr>
<tr>
<td><em>Nostoc mucorum</em></td>
<td>53.0°C</td>
<td>56.0°C</td>
<td>54.0°C</td>
</tr>
<tr>
<td><em>Plectonema</em> sp.</td>
<td>-</td>
<td>57.0°C</td>
<td>54.0°C</td>
</tr>
</tbody>
</table>

### 4.5 Data analysis

The extracted RNA concentration was normalized due to the different cell density of different biological replicates and calculated using the following equation: $C = \frac{c_l}{\alpha} \times 0.2$, where $C_l$ was the original extracted RNA concentration, $\alpha$ was optical density of culture when harvested. Statistical analyses were conducted in SAS (v. 9.3, SAS Institute Inc.). Tukey’s test ($\alpha=0.05$) was used to analyze the differences in extracted RNA concentration of target species among different extraction methods.
CHAPTER 5 – RESULTS AND DISCUSSIONS

5.1 Evaluation of RNA extraction yield and purity

For all the three methods investigated, RNA was extracted from approximately $10^9$ cells of each culture. The yield and purity of extracted RNA were determined by a wavescan of absorbance ranging from 220 nm to 340 nm (Figure 9). The position and magnitude of these peaks and valleys give indications of sample composition and purity that helps to identify possible contamination sources. A typical spectrophotometric profile of purified RNA contains a valley at around 230 nm and a peak at around 260 nm [119]. Any additional peaks indicate possible contamination in an extracted total RNA sample. Wavescans from samples using the three methods from all species match the criteria of purified RNA except extracted RNA using Qiagen and TRIzol method from *N. muscorum*. No 260 nm peak and no 230 nm valley was observed from *N. muscorum* sample using the Qiagen method, suggesting extremely low yield of RNA but relatively high proportion of chaotropic salt contamination absorbed at 230 nm. The concentration of nucleic acids was estimated according to the value of the 260 nm absorbance, while the purity of each sample was determined by the $A_{260}/A_{280}$ and $A_{260}/A_{230}$ ratios (Table 7, Figure 9 and Figure 10).

The extracted RNA concentration when using CTAB method (897 ng/μl) was about 12 times higher than that when using Qiagen method (69 ng/μl) for *Nostoc muscorum*, and it was 3 times for *Synechocystis* sp. comparing CTAB result (616 ng/μl) to Qiagen (205 ng/μl). The highest yields were achieved using the CTAB method for *Plectonema* sp. --- averaging 2585 ng/μl. The CTAB method always gave the highest yield when compared to the other methods.
Figure 9. Extraction contamination analysis.
Absorption spectra in the UV-region for purified RNA from three species using 3 different extraction methods: (A) Syenochystis sp., (B) Plectonema sp., (C) N. muscorum. From all obtained RNA samples, 1 µl was analyzed using NanoVette Beckman Coulter Life Science UV/Vis spectrophotometer. The resulting curves shown are averaged from values obtained from corresponding extraction method. A valley at around 230 nm and a peak at around 260 nm are expected for a “pure” RNA sample.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Qiagen</th>
<th></th>
<th></th>
<th>TRizol</th>
<th></th>
<th></th>
<th>CTAB</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RNA Conc. (ng/µl)</td>
<td>A_{260}/A_{280}</td>
<td>A_{260}/A_{230}</td>
<td>RNA Conc. (ng/µl)</td>
<td>A_{260}/A_{280}</td>
<td>A_{260}/A_{230}</td>
<td>RNA Conc. (ng/µl)</td>
<td>A_{260}/A_{280}</td>
</tr>
<tr>
<td><em>Synechocystis sp.</em></td>
<td>205</td>
<td>1.897</td>
<td>1.372</td>
<td>437</td>
<td>2.032</td>
<td>2.153</td>
<td>616</td>
<td>2.079</td>
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<tr>
<td>PCC 6803</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Plectonema sp.</em></td>
<td>579</td>
<td>1.991</td>
<td>2.075</td>
<td>718</td>
<td>2.041</td>
<td>2.31</td>
<td>2585</td>
<td>2.127</td>
</tr>
<tr>
<td><em>Nostoc muscorum</em></td>
<td>69</td>
<td>1.899</td>
<td>0.478</td>
<td>394</td>
<td>2.058</td>
<td>1.178</td>
<td>897</td>
<td>2.049</td>
</tr>
</tbody>
</table>
Figure 10. Extracted RNA yield and purity. Yield and absorbance ratios for the different extraction method from the three species were determined. (A) *Syenochocystis* sp., (B) *Plectonema* sp., (C) *N.muscorum*. For each sample, 1 µl was analyzed using NanoVette Beckman Coulter Life Science UV/Vis spectrophotometer. The bar chart shown are the averages of three repetitions and the standard deviations from values obtained for each extraction method. The columns do not share the same bar are significant different (p<0.05). The expected 260/280 ratio is 2.0±0.2, while the expected 260/230 ratio should be over 2.0.
with the same strain, while the Qiagen methods always resulted in the lowest yield, and the yield of the TRIzol method fell in between. This variance was likely due to the high cell lysis efficiency of CTAB extraction buffer when used to purify nucleic acids from organisms which produce large quantities of polysaccharides, like plants and some gram-negative bacteria [102, 112, 113, 115]. As expected, the yield and quality (especially 260/230 ratio) of extracted RNA from *N. muscorum* were relatively low compared to the other two species, due to its complicated multi-plane three dimensional architecture with spreading film, despite the sheath surrounding the vegetative cells during the growth and high polysaccharides contents in cells [38, 89, 111]. Surprisingly the yields of *Plectonema* sp. using all methods were higher than that of *Synechocystis* sp.. In particular, the RNA concentration of *Plectonema* sp. using CTAB method was over four times of that of *Synechocystis* sp., and *N. muscorum* using CTAB method also produced more RNA than it did for *Synechocystis* sp., although yields from *N. muscorum* using the other two methods are still low. This result suggested that the application of CTAB greatly promotes the extraction efficiency compared to the other two methods using chaotropic solutions.

For all the three strains, 260/280 ratios from all methods were around 2.0 (±0.2) – the expected value for a “pure” RNA sample (Table 7 and Figure 10). The samples extracted from *Synechocystis* sp. and *N. muscorum* using the Qiagen method had a relatively lower 260/280 ratio, close to 1.9, but still fell into the range of pure RNA (pH 1.8-2.0) since the RNA was measured in RNase-free water instead of TE (pH 8.0) [126].

However, the 260/230 ratio of RNA samples extracted from *Synechocystis* sp. using the Qiagen method and those from *N. muscorum* using both the Qiagen and TRIzol methods
were significantly lower than 2.0, indicating high level of contamination with peptides, phenols, aromatic compounds or carbohydrates [119]. The extracted RNA of *N. muscorum* using the Qiagen kit had the lowest value (0.478), indicating considerable guanidinium thiocyanate contamination carried over from Qiagen RLT buffer (Table 7 and Figure 10). Interestingly, according to the wavescans (Figure 9), the absolute O.D. values at 230 nm of CTAB samples were significantly higher than those of Qiagen and TRIzol samples, suggesting that the low 260/230 ratios are more related to the yields other than the amount of carryover contamination itself.

**5.2 Verification of extracted RNA integrity**

To verify the integrity of the RNA samples, triplicates from each method were analyzed by running gel electrophoresis on a 1.2% agarose gel with 0.5× TBE buffer (Figure 11). All patterns of rRNA were observed. Four rRNA fragments were observed consistently from the extracted total RNA from *Synechocystis* sp. and *N. muscorum*. Though most gram-negative bacteria only show three rRNA fragments, 5S, 16S and 23S rRNA, it is reported that 23S rRNA fragment from certain cyanobacteria strain is cleaved generating two molecules, one larger and one smaller than 16S rRNA [127]. With the exception of one sample of *Plectonema* sp. extracted using the Qiagen method showing minor degradation, all the other extracted RNA samples were intact.

Visible high molecular weight bands were observed from *Synechocystis* sp. RNA extracted using the CTAB method and from *N. muscorum* RNA extracted using the TRIzol and CTAB methods indicating putative genomic DNA contaminations [105]. Since some level of genomic DNA contamination was expected but not necessarily detectable via
electrophoresis for all extraction methods [36], DNase I treatment to eliminate the genomic DNA contamination is necessary to ensure accurate downstream application results.

A.

B.

C.

Figure 11. Gel electrophoresis of total RNA extracted from cyanobacteria using different methods. Total RNA extracted from three cyanobacterial strains *Synechocystis* sp. (A), *Plectonema* sp. (B) and *N. muscorum* (C) using different extraction methods were electrophoresed on a 1.2% agarose gel in 0.5x TBE buffer. Four rRNA fragments under low molecular weight region (<2kb) should be observed as indications of integrated of extracted total RNA.
5.3 RT-PCR analysis

Reverse transcription (RT) is used to determine if the extracted RNA was suitable for common downstream applications since it is known to be inhibited by endonucleases, exonucleases and photosynthetic pigments [46]. Both gene-specific RT-PCR and fingerprint RT-PCR were performed by using 1 µg of RNA from each extraction method as a template to synthesize and amplify fragments of phycocyanin transcriptional regulator gene (cpcA) [125], 16S-23S ribosomal RNA and its internal transcribed spacer (rRNA ITS) [110], Fe subunit protein of Nitrogenase encoding gene (nifH) [124] and cyanobacterial highly iterated palindrome (HIP) [117]. Prior to RT, the obtained total RNA was treated with DNase I by using the TURBO DNA-free™ Kit (Ambion, Cat.# AM1907) according to the manufacturer’s instructions to avoid the interference of genomic DNA contamination. The RT-PCR products were analyzed by running a 2.0% agarose gel electrophoresis (Fig. 12).

It is believed that bacteria cells or debris, proteins, polysaccharides and any residual buffer that carried over during the extraction process would inhibit the amplification of nucleic acids by PCR by physical effects such as making the DNA/RNA target unavailable to the polymerase/reverse transcriptase [120, 128]. Unavailability of target or primer DNA by non-specific blocking or sequestration inhibits amplification or cause misleading band variations during typing based on PCR or RT [128]. According to electrophoresis results of gene-specific RT-PCR (Figure 12 A, B and C), when priming with cpcA primer [125] using the equal amount of total RNA, the RT products from CTAB methods gave the highest yields while TRIzol gave the lowest yields suggesting that some high level of contamination from TRIzol inhibiting activity of enzymes in RT-PCR (Figure 12 A) [128]. When priming with
Figure 12. RT-PCR suitable RNA extraction. Agarose gel separation of RT-PCR products. For each PCR, 1 µg of RNA was used as template for RT primed with (A) cpcA gene primer (not specific for *Plectonema*), (B) nifH gene primer (not specific for *Synechocystis*), (C) 16S rRNA, 23S rRNA and ITS primer and (D) Highly iterated palindrome primer. The RNA samples of each strain were extracted from Qiagen method, TRIzol method and CTAB method respectively. And for HIP primer, an extra negative control using non-RT total RNA was used in the 4th lane of each strain.
rRNA ITS primer (Figure 12 C) [110], two main PCR products were observed, indicating the presence of multiple, heterologous rRNA operons [110]. In *Synechocystis* sp., product from TRIzol method had relative lower yield compared with the others. In *N. muscorum*, the PCR product from Qiagen method showed the lowest yield, whereas *Plectonema* sp., the PCR products were almost identical. The results of RT-PCR using the rRNA ITS primer were consistent with the results we got from NanoDrop (Figure 10): the low 260/230 ratio of extracted RNA of *N. muscorum* from Qiagen negatively impacted the RT procedure. While TRIzol Reagent was not recommended to be used to extract total RNA for sensitive downstream applications like real time PCR or microarray. All the three methods were able to generate RT suitable RNA from *Plectonema* sp.. When priming with *nifH* primer (Figure 12 B) [129], extra products were observed from the samples using TRIzol method further confirming that TRIzol could not only weaken the activity of enzymes involving in reverse transcription, but also interfere with the RNA profiling results.

The electrophoresis result of RT-PCR using the HIP primer (Figure 12 D) showed consistent species-specific fingerprint. However, no RT product was observed from *Plectonema* sp. using the Qiagen method suggesting the inconsistency of the Qiagen method when performing the RNA extraction from cyanobacteria.

Both gene-specific and HIP primer RT-PCR have demonstrated the reverse transcription suitability of extracted total RNA using CTAB method, which confirmed the spectrophotometry results presented earlier (Figure 9 and Figure 10).
5.4 Critical modifications and principles behind the method

The mainly difference among the three methods is the basic chemistry used when performing beads beating. Both the RLT buffer used in the Qiagen RNeasy kit and the TRIzol Reagent are high concentrated guanidine isothiocyanate buffer developed from Chomczynski’s single-step method [130]. The difference was that the Qiagen kit uses silica membrane binding technology while the TRIzol uses the acidic phenol chloroform extraction to keep total RNA in the upper aqueous phase and most of the DNA and protein remaining either in the interphase or lower organic phase. CTAB is demonstrated to overcome the problem of polysaccharide and phenolic compounds contamination for RNA extraction in microalgae [115]. 2-mercaptoethanol is added to the extraction buffer (RLT in the Qiagen method and CTAB extraction buffer in the CTAB method) to inhibit endogenous RNase activity [131]. Substituting BCP for chloroform in the TRIzol protocol and the CTAB protocol does not affect the quality of isolated RNA, and its employment as the phase separation reagent may decrease the possibility of contaminating RNA with DNA [132]. Precipitation of RNA with isopropanol in the presence of the High Salt Precipitation Solution (described above in the TRIzol protocol) is proven to prevent polysaccharide and proteoglycan contamination of RNA [133]. However, the low 260/230 ratio still indicated high polysaccharide contamination due to the cell wall structure differences between cyanobacteria and animal tissues. High concentration of LiCl is used in the CTAB protocol to effectively precipitate RNA out of DNA [101, 115].
5.5 Cost analysis for different methods

The cost per sample for different protocols is estimated based on the manufacturer’s suggested retail prices for molecular biology grade reagents in the minimal quantities available in the U.S. during August 2012. For RNeasy Mini Kit (Qiagen, cat. # 74104), the cost is $5.14. For the TRIzol protocol, the cost (TRIzol Reagent, Invitrogen, cat. # 15596-026) is $3.14. For the CTAB protocol, the cost per sample is around $0.89 (Table 8), which is 28.3% of the cost of the TRIzol method, and only 17.3% of the cost of the Qiagen method.

Table 8. Cost analysis of CTAB RNA extraction protocol

<table>
<thead>
<tr>
<th>Method</th>
<th>Chemicals/product</th>
<th>Retailer</th>
<th>Cat. no.</th>
<th>MSRP</th>
<th>Quantity</th>
<th>Price per rxn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexadecyltrimethylammonium</td>
<td>Bromide (CTAB)</td>
<td>Fisher</td>
<td>AC22716-1000</td>
<td>$43.82</td>
<td>100 g</td>
<td>$0.009</td>
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<tr>
<td></td>
<td>Tris-HCl</td>
<td>Promega</td>
<td>H5121</td>
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<tr>
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<td>NaCl</td>
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<td>Fisher</td>
<td>S312-500</td>
<td>$302.66</td>
<td>500 g</td>
<td>$0.005</td>
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<td></td>
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<tr>
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<td>Invitrogen</td>
<td>15596-026</td>
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<td>100 ml</td>
<td>$3.136</td>
</tr>
</tbody>
</table>
CHAPTER 6 – CONCLUSIONS AND FUTURE WORKS

Cyanobacteria are morphologically diverse, produce a significant amount of polysaccharides, and are rich in nucleases and enzymatic reaction inhibitors [46-48, 120, 128]. All these characteristics make cell lysis and nucleic acid purification difficult [134, 135]. In this work, a RNA extraction protocol using a simple extraction solution containing CTAB was developed. The new method is proven to be able to isolate high quality and quantity total RNA from three morphologically distinct cyanobacteria strains: *Synechocystis* sp. PCC 6803, *Plectonema* sp. UTEX 1541 and *Nostoc muscorum* UTEX 1037.

Two traditional protocols previously used to extract total RNA from cyanobacteria using the Qiagen RNeasy Mini Kit [107] and TRIzol Reagent [36] respectively were also performed. The extracted total RNA using the CTAB method show significantly improvement in both quality and quantity compared to the RNA extracted using Qiagen and TRIzol method.

It is generally thought the complicated cell wall structure would greatly hinder the cell lysis, however the highest yield was achieved by the ensheathed filamentous *Plectonema* sp. using CTAB method instead of unsheathed unicellular *Synechocystis* sp.. Two possible reasons could factor this result: 1) the cell density of filamentous *Plectonema* sp. is higher than expectation, thus more cells were involved in the extraction; 2) “false branching” doesn’t occur to *Plectonema* sp., which means a string of cells may share a single layer of sheath or surface layer, which greatly promotes the disruption efficiency.
The newly developed CTAB RNA extraction method is of low cost compared to the other two methods, but is still able to give much higher yield and quality total RNA from the three selected cyanobacteria strains. Compared to the other RNA extraction methods that are also low-cost and efficient [36, 105], the CTAB method uses the commercial available acidic phenol: chloroform solution instead of the hot saturate phenol or phenol crystals, both which are less safe to use.

RT-PCR with both gene-specific primer and HIP primer using the extracted total RNA as a template shows that the CTAB method successfully eliminated the interference of PCR inhibitors such as polysaccharides, which negatively impact reverse transcriptase and polymerase activity during RT-PCR [120, 128].

The success in total RNA extraction from morphologically distinct cyanobacteria using the CTAB method is fundamental for our future research on the cyanobacterial PHA metabolism. Furthermore, since the three selected strains were morphologically distinct, and from three different cyanobacterial structural sub-groups, the CTAB method might be further applied to high quality total RNA extraction from all cyanobacteria.
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VITA

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