PRODUCTION OF NITROGEN-BASED PLATFORM CHEMICAL: CYANOPHYCIN BIOSYNTHESIS USING RECOMBINANT *ESCHERICHIA COLI*

by

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Abstract

Synthesis of chemical derivatives from finite fossil fuels requires considerable energy inputs and leaves an undesirable environmental footprint. The emerging biorefinery approach leads to sustainable processing of biomass into a wide spectrum of bio-based products, catering to food, feed, chemicals, materials, and bioenergy industries.

Cyanophycin (multi-L-arginyl-poly-L-aspartic acid, CGP) is a non-ribosomally synthesized reserve polypeptide, which consists of equimolar amounts of arginine and aspartic acid arranged as a polyaspartate backbone and arginine as the side chain. Cyanophycin is a source of the constituent N-functionalized platform chemical, which can be further processed into many other chemicals of importance. It can be hydrolyzed in mild condition to its constituent amino acids - aspartic acid and arginine. These amino acids may be utilized directly in food and pharmaceutical applications. Based on the chemical structure of these amino acids and the presence of functionalized nitrogen-containing groups, it is conceivable that a number of industrial chemicals can be synthesized, for example: 1, 4-butanediamine， a co-monomer in the production of nylon-4, 6. Other chemicals which could be obtained from cyanophcyin, that are currently prepared from fossil resources, include 1,4-butanediol and urea. Cyanophycin can also be hydrolyzed to a derivative with reduced arginine content or even to poly-aspartic acid, and used as a biodegradable substitute for synthetic polyacrylate in various technical process, such as water treatment (water softeners) and plastics.

Cyanophycin is produced by most cyanobacteria in nature; however, these microbes are not suitable for large-scale production due to slow growth and low polymer content. Biosynthesis of cyanophycin is catalyzed by a single enzyme - cyanophycin synthetase (CphA), which is encoded by cyanophycin synthetase structure gene (*cphA*). The *cphA* gene can be expressed in

several bacteria and plants. *E.coli* is one of the most commonly used bacterial hosts for the production of recombinant proteins. The recombinant culture has the ability to produce considerably large quantities of cyanophycin in a shorter period of time compared to cyanobacteria.

Genome of *Anabaena variabilis* ATCC 29413 has been sequenced, and it contains the structural gene (*cphA*) for cyanophycin synthetase. The native enzyme-cyanophycin synthetase from this cyanbacterium culture had been purified and it consists of identical subunits of 98kDa. Polymerization of the amino acids to cyanophycin *in vitro* is dependent on the presence of ATP, K^+, Mg^{2+}, a (cyanophycin) primer and a thiol reagent such as β-mercaptoethanol in the reaction mixture. Our research is the first time that *cphA* gene from *A. variabilis* ATCC 29413 was cloned into *E.coli*. The 2.7 kb *cphA* gene was amplified by PCR, ligated to the vector pET45b+ and cloned into BL21 (DE3) pLysS and BL21 (DE3).

Characterization of cyanophycin was performed by SDS-PAGE, HPLC, mass spectrometry and amino acid analysis. Results showed that protein with molecular weight of 21.5 to 31 kDa did not match any *E.coli* proteins when compared with *E.coli* protein data base, thereby showing expression of a protein foreign to host strain. Amino acids analyses showed that the cyanophycin produced by recombinant *E.coli* contained aspartic acid and arginine, and small amount of lysine, in the ratio: 1.05: 1: 0.2 (mass basis), thereby confirming cyanophycin biosynthesis.

Experiments for high cyanophycin synthesis was performed at shake flask and 2-L fermentor level using recombinant BL21 (DE3) pLysS, LB broth as carbon and nutrient source, and casamino acids as primer. The maximum yield of cyanophycin obtained in flask level was 7.6% of cell dry mass, and the yield increased to 12.6% of cell dry mass at 2-L fermentor level.

Cyanophycin is also referred to as “structural granules” because of substructures visible in electron micrographs. Phase contrast photomicrograph was able to depict cyanophycin inclusions in the cytoplasm, and transmission electron microscopy depicted finer details inside cell after IPTG induction.

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CHAPTER 1 - Introduction

Cyanophycin (CGP) was identified more than 100 years ago. The Italian botanist, Borzi (1887) introduced the term "cyanoficina" for a nitrogen rich compound in the cytoplasm of cyanobacteria (also called blue-green algae). Cyanophycin granules have been found in most but not all species of cyanobacteria. Only recently, cyanophycin granules have been reported to be found in non-cyanobacterial, heterotrophic bacterium, namely, *Acinetobacter calcoaceticus* strain ADP1, *Beijerinckia derxii* and so on (Miyasaka *et al*., 2003; Krehenbrink *et al*., 2004).

In 1971, Simon reported the extraction and characterization of cyanophycin granules from cyanobacterium *Anabaena cylindrica*. It is an unusual polypeptide, which consists of only two amino acids: L-arginine and L-aspartic acid, in the ratio of 1:1 and is highly polydispersed with molecular weight ranging from 25 to 100 kDa (Simon, 1971; Simon and Weathers, 1976). It is now well-established that cyanophycin consists of equimolar amounts of aspartic acid and arginine arranged as a poly-aspartic acid backbone to which arginine residues are linked to the β-carboxyl group of each aspartic acid by its α-amino group (Mooibroek *et al.*, 2007). Figure 1.1 showed the chemical structure of cyanophycin monomer. According to its chemical structure, cyanophycin is a zwitter-ion molecule with a predicted isoelectric point (IP) of about 7. Simon and Weathers (1976) reported that by polyacrylamide gel isoelectric focusing, a main band with an IP equal to 4.75 and a minor band with IP equal to 6.1 were observed for cyanophycin from *Anabaena cylindrica*. The minor band was cyanophycin with a 1:1 molar ratio of Asp:Arg, whereas the main band at pH 4.75 may have lost some arginyl residues.

Cyanophycin is highly resistant against hydrolytic cleavage by proteases, such as trypsin, pronase (a commercial available mixture of proteinase), pepsin, carboxypeptidases B, carboxypeptidase C, and leucin-aminopeptidase and arginases (Mooibroek *et al*., 2007).

However, cyanophycin can be degraded by an extracellular enzyme, which is cyanophycinase encoded by a structure gene *cphB* (Richter *et al*., 1999). When cyanophycin-rich cultures are transferred to nitrogen-deficient media, the polymer is rapidly degraded and most probably used as a source of nitrogen (Allen and Hutchinson, 1980).

Cyanophycin is a source for the constituent N-functionalized precursor chemicals. The direct application of cyanophycin itself is unknown right now; however, as a platform chemical it can be converted to many other important chemicals. Figure 1.2 shows the application of this polymer. Cyanophycin can be hydrolyzed to its constituent amino acids: aspartic acid and arginine. These amino acids may be utilized directly in food and pharmaceutical applications. Based on the chemical structure of these amino acids and the presence of functionalized nitrogen-containing groups, it is possible to produce a number of industrial chemicals, for example: 1, 4-butanediamine, which is derived from petrochemistry and is currently used as a co-monomer in the production of nylon-4, 6. Other chemicals that could be obtained from cyanophycin, but are currently prepared from fossil resources include 1, 4-butanediol and urea (Mooibroek *et al*., 2007). Cyanophycin also can be hydrolyzed to a derivative with reduced arginine content or even to poly-aspartic acid, and used as a biodegradable substitute for synthetic polyacrylate in various applications, such as water treatment (water softerners) and plastics (Joentgen *et al*., 1998).

Cyanophycin has unusual solubility properties. It is insoluble at neutral pH and physiological ionic strength but soluble in diluted acids and bases (Simon, 1971). Methods were developed for the isolation of cyanophycin by taking advantage of its unusual solubility properties. 0.1 M HCl was applied to extract cyanophycin and then titrate at pH 7 to precipitate it. The characterization of cyanophycin is not easy, since this unique polymer is highly-

polydispersed and no commercial standard are currently available. According to literature, characterization of cyanophycin was performed mainly by SDS-PAGE, HPLC and Mass spectroscopy (Ziegler *et al*., 2002; Elbahloul *et al*., 2005b).

Cyanophycin can be produced by most cyanobacteria in nature. It has been proposed that cyanophycin is a dynamic nitrogen reservoir, the content of cyanophycin in the cell fluctuating according to the needs of cell (Carr *et al*., 1988). Cyanophycin is synthesized without the participation of ribosomes or messenger RNA as template. The polymerization reaction is catalyzed by a single enzyme referred to as cyanophycin synthetase (CphA), which is encoded by the *cphA* gene (Ziegler *el al.*, 1998; Füser and steinbüchel, 2007). Cyanophycin synthetase from *Synechocystis* sp. PCC 6308 (Aboulmagd *et al*., 2000; Aboulmagd *et al*., 2001a), *Anabaena variabilis* ATCC 29413 (Ziegler *et al*., 1998) and *Synechococcus* sp. MA19 (Hai *et al*., 1999, 2000) has been purified and characterized.

The cyanobacteria are not suitable for large-scale production of cyanophycin because of the low polymer content and slow growth, and therefore, sufficient amount of cyanophycin are not available. Actually, in rapidly growing cultures of cyanobacteria, the cyanophycin content is usually less than 0.5% of the cellular dry mass (Simon, 1973a). However, under some conditions, such as, sulfate or phosphate deprivation, or presence of inhibitors of ribosomal protein synthesis, the polymer may be present at more than 10% of the cellular dry weight (Sutherland *et al*., 1979). Currently, other systems for large scale production of cyanophycin are being developed. The approach is based on the heterologous expression of cyanophycin synthetase in bacteria and plants. Ziegler *et al*. (1998), Frey *et al*., (2002), Aboulmagd *et al*., (2000, 2001b) have cloned *cphA* gene from cyanobacteria into *E.coli* and other heterotrophic bacteria. Large amount of cyanophycin production (up to 26% of dry cell mass) is observed.

However, such high content are only reached in complex culture media, which is expensive. Low-cost agricultural by-products as substitute for complex media need to be evaluated in order to decrease the cost; however, not much work has been done so far. Elbahloul *et al*., (2005a) evaluated protamylasse, a residual compound occurring during the industrial production of starch from potatoes as a substitute substrate for production of cyanophycin. A maximum CGP content of 28% (wt/wt) per cell dry matter was obtained in 6% (vol/vol) protamylasse medium at an initial pH of 7.0 within a cultivation period of only 24 h. Cyanophycin from recombinant strains harboring *cphA* exhibit a much lower molecular weight ranging from 25 kDa to 30 kDa (Mooibroek *et al*., 2007).

The objectives of the present work are to develop a recombinant E.coli strain, which harbors cphA gene from *Anabaena variabilis* ATCC 29413, and devise an efficient bioprocess for production of cyanophycin using recombinant *E.coli* strain.

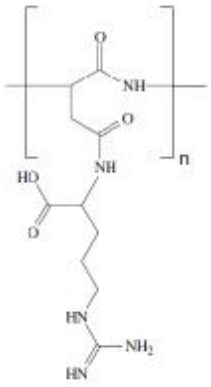


Figure 1.1 Chemical structure of cyanophycin monomer (Mooibroek *et al*., 2007)

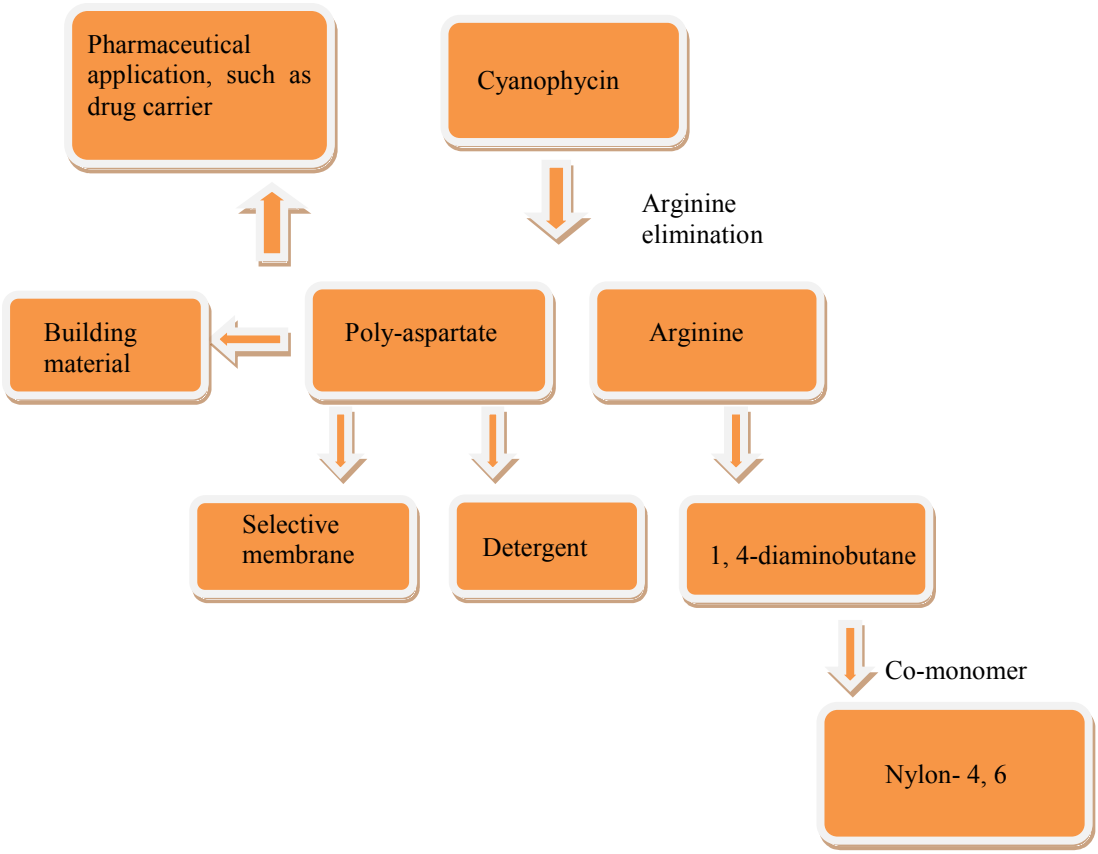


Figure 1.2 Derivation of important nitrogen-based chemicals from cyanophycin

CHAPTER 2 - Production of cyanophycin from *Anabaena variabilis* ATCC 29413

Introduction

Cyanobacteria are oxygenic photosynthetic prokaryotes that can be found in diverse habitats, including some stressful environmental conditions. One of their survival mechanisms is to accumulate reserve material such as a nitrogen-rich compound (Allen, 1984). Many cyanobacteria produce a characteristic sub-cellular inclusion known as cyanophycin, which acts as a dynamic, fluctuating reservoir for the assimilation of nitrogen (Allen and Hutchison, 1980; Allen, 1988). Under certain environmental conditions, synthesis of cyanophycin can be induced; for example, by high light or CO_2 supply, sulfur or phosphorus starvation, addition of inhibitors of ribosomal protein synthesis, and addition of arginine (Allen *et al*., 1980).

Anabaena variabilis ATCC 29413 is a filamentous cyanobacterium (Figure 2.1). Under nitrogen-limiting conditions, vegetative cells differentiate into heterocyst at semi-regular intervals along the filaments. Heterocysts are cells that are terminally specialized for nitrogen fixation. Light and electron microscopy observations have shown that cyanophycin granules are present in older cultures and are particularly abundant in spores, but are lacking in actively growing cultures (Fogg, 1951; Miller *et al*., 1971; Neilson *et al*., 1971). Carr (1988) observed that cyanophycin synthetase and the degrading enzymes were more active in heterocysts of nitrogen-fixing *Anabaena* species than in the vegetative cells; his hypothesis made cyanophycin an integral part of the interplay between nitrogen and carbon metabolism in cyanobacteria.

Biochemical studies on cyanophycin accumulation in cyanobacteria include treating cells with a protein synthesis inhibitor, for example, chloramphenicol, to enhance the amount of cyanophycin accumulation within the cell. Culturing cyanobacterium *Synechocystis* 6803 in

photobioreactors, the appreciable accumulation of polymer of about 5% on a dry weight basis was achieved only when L-arginine and chloramphenicol were added to stationary phase culture (Hai *et al*., 2000; Oppermann-Sanio and Steinbüchel, 2002).

The objective of this study was to evaluate the effect of chloramphenicol addition on growth of *A. variabilis* ATCC 29413 and the production of cyanophycin. Molecular weight of cyanophycin produced by *A. variabilis* ATCC 29413 was estimated by HPLC.

Materials and methods

ATCC medium # 616 (BG-11) was prepared in our Bioprocessing Laboratory, BIVAP, Grain Science & Industry Department, KSU. The medium consisted of: $NaNO_3$ 1.5 g/l, K_2HPO_4 0.040 g/l, $MgSO_4.7H_2O$ 0.075 g/l, $CaCl_2.2H_2O$, 0.036 g/l, citric acid 6.0 mg/l, EDTA 1.0 mg/l, Na_2CO_3 0.020 g/l, Trace metal mix A5 1.0 ml (H_3BO_3 2.86 g/l, $MnCl_2.4H_2O$ 1.81 g/l, $ZnSO_4.7H_2O$ 0.222 g/l, $Na_2MoO_4.2H_2O$ 0.391 g/l, $CuSO_4.5\ H_2O$ 0.0790 g/l, $Co(NO_3)_2.6\ H_2O$ 49.4 mg/l); and adjusted to final pH 7.1.

A. variabilis ATCC 29413 (Figure 2.1) was obtained from American Type Culture Collection (ATCC), VA, USA.

Fermentation conditions

A 5-L BIOFLO 110 fermentor (New Brunswick Scientific, Edison, New Jersey, USA) was used in the experiments (Figure 2.2). The fermentor has a fully automated control panel and autoclavable. A deionized water system was installed to meet the requirements for built-in steam generator and vessel jacket during sterilization. About 4 L BG-11 medium was collected and the fermentor-containing medium was sterilized at temperature 121 ºC for 15 min prior to inoculation. Fermentation was performed at 30 ºC. The fermentor was inoculated with *A.*

variabilis ATCC 29413 at a 5 % inoculum (200 ml of culture to 4 L fresh medium). Culture was illuminated by cool white flurescent lamp (Glare reducing light TL1000, St Paul, MN, USA). Air was pumped at 0.6 air/volume of fermentation medium/minute (VVM) to supply CO_2. The agitation speed was maintained at 60 RPM. Chloramphenicol was added to the exponentially growing culture in 5 L fermentor to a final concentration of 10 µg /ml.

Determination of cell dry mass

Duplicate 5-ml sample of cell culture was centrifuged at 8000×g for 20 min (Sorvall Superspeed centrifuge RC2-B, Newtown, CT, USA), washed with sterile saline solution, and dried at 60 ºC (Isotemp oven 6250, Fisher Scientific, USA) for 24 hours until constant weight was obtained.

Cyanophycin isolation

Isolation of cyanophycin was followed as previously described by Simon (1973a), using a modified protocol. Approximately 0.3 g lyophilized cells in late exponential phase were resuspended in 10 ml distilled water. Cells were broken using sonicator (Mixsonix sonicator S-4000, Division of Qsonica, LLC, USA) at 50 amplitude at 1 min intervals separated by cooling on ice for 1 minute, which was continued until all cluster cells disintegrated to single cells. Triton X-100 was then added to a final concentration of 0.5% and the suspension was sonicated for two more 1 min periods. The detergent containing cell suspension was stirred overnight at 4 ºC, and was then centrifuged for 10 min at 30,000×g (Sorvall Superspeed centrifuge RC2-B, Newtown, CT, USA). The supernatant was discarded and the pellets were washed five times with distilled water to remove solubilized material and the Triton X-100. Then, pellets were resuspended in 10 ml 0.1 M HCl, centrifuged for 10 min at 30,000×g. Supernatant fluid was

removed and titrated to pH 7.0 using 1.0 M NaOH. Cyanophycin was precipitated by centrifugation, washed with double distilled water and lyophilized. The content of crude cyanophycin was expressed as % of cell dry matter.

HPLC analysis

Polymer extracted from two-week culture was dissolved in 0.1 M HCl. 1 ml of sample was filtered through a 0.45 syringe filter, and analyzed using a HPLC system (CBM-20A system, Shimadzu, Columbia, MD, USA) equipped with a size extrusion column (Biosep SEC-S 2000), a UV detector (Shimadzu SPD-M20A) and a refractive index detector (Shimadzu RID-10A). Samples were detected at 210 nm. 0.1 M HCl solution was used as mobile phase at flow rate of 1.00 ml/min. The HPLC column was operated at 40 ºC. Calibration was done with standard size protein purchased from Invitrogen (Invitrogen Corporation, CA, USA).

Results and discussion

Effect of chloramphenicol on growth and cyanophycin production of A. variabilis ATCC 29413

Chloramphenicol had a negative effect on the growth of *A. variabilis* ATCC 29413 (Figure 2.3). At the end of fermentation, cell dry mass was 4.18 g/l without chloramphenicol addition; however, cell dry mass deceased to 2.43 g/l at the end of fermentation when chloramphenicol was added at day 7. The content of crude cyanophycin isolated from culture without chloramphenicol addition was 0.27 % of cell dry matter and the content of crude cyanophycin isolated from culture with chloramphenicol addition increased to 2.43 %, which corresponded to results reported by Simon (1973b). They found out that the addition of

chloramphenicol inhibited the growth of *Anabaena cylindrica*, while, the cyanophycin granule increased markedly from 0.05 % of the cell dry weight to 2.4 %. It was conceivable that the inhibition of ribosome activity may direct the amino acids to follow the non-ribosomal synthesis pathway, which increased the cyanophycin content, while some ribosomally synthesized protein for cell growth was inhibited and therefore caused decrease in growth rate. The yield was low and did not exceed 5% yield reported by Oppermann-Sanio and Steinbüchel (2002), which may due to lack of arginine in the medium.

Molecular weight of cyanophycin analysis

The Molecule weight of cyanophycin from *A. variabilis* ATCC 29413 was about 21.5-31 kDa (Figure 2.5) when compared with standard protein (Figure 2.4). Each peak represented a protein with a specific molecular weight (Table 2.1). The smaller molecule had longer retention time than bigger molecule, because the stationary phase particles had small pores and/or channels which will allow molecule below a certain size to enter. The large particles were therefore excluded from these pores and channels and they were eluted from the column earlier than small particles. Although the molecular mass of the cyanobacteria polymer ranged from 25-100 kDa, it was found to elude from the column in one sharp peak at retention time of 11.540 min.

Microscopy study

Cyanophycin is also referred to as “structural granules” because of substructures visible in electron micrographs (Lang, 1968), which were variable in shape and size and had a radiating pattern of substructure. Contrast-phase micrograph (Figure 2.6) (Hitachi S-3500N scanning electron microscope, Tokyo, Japan) showed the heterocyst cell marked by an arrow in which

cyanophycin granules were formed. TEM provided morphological information about the size, shape and arrangement of organelles inside the cells and the relationship among microorganisms in areas a few nanometers in diameter (Mariné *et al*., 2004). Transmission electron micrograph of *A. variabilis* ATCC 29413 (Figure 2.7) showed that cyanophycin granules were the structured granules in the cell.

Conclusion

The production of cyanophycin from *A. variabilis* ATCC 29413 was low without addition of chloramphenicol (only 0.27%), and after addition of chloramphenicol, the yield of cyanophycin increased to 2.43% of cellular dry mass, whereas the growth was inhibited. At the end of cultivation, cell density decreased to 2.57 g/l with chloramphenicol addition. Cyanobacterium is not suitable for cyanophycin production because of its slow growth and low molecular content. To improve cyanophycin yield, our next goal was to clone *cphA* gene from *A. variabilis* ATCC 29413 into high expression, fast growing *E.coli* host cells.

Table 2.1 Molecular weights of standard protein corresponding to retention time

Peak #	Retention time (min)	Molecular weight (kDa)
1	5.470	200
2	8.361	116.3
3	8.751	97.4
4	9.961	66.3
5	10.185	55.4
6	10.517	36.5
7	10.833	31.0
8	11.269	21.5
9	12.959	14.4
10	13.650	6.0

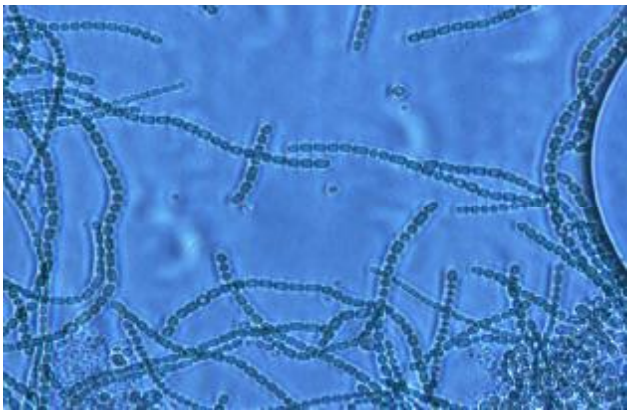


Figure 2.1 ***A. variabilis*** **ATCC 29413**

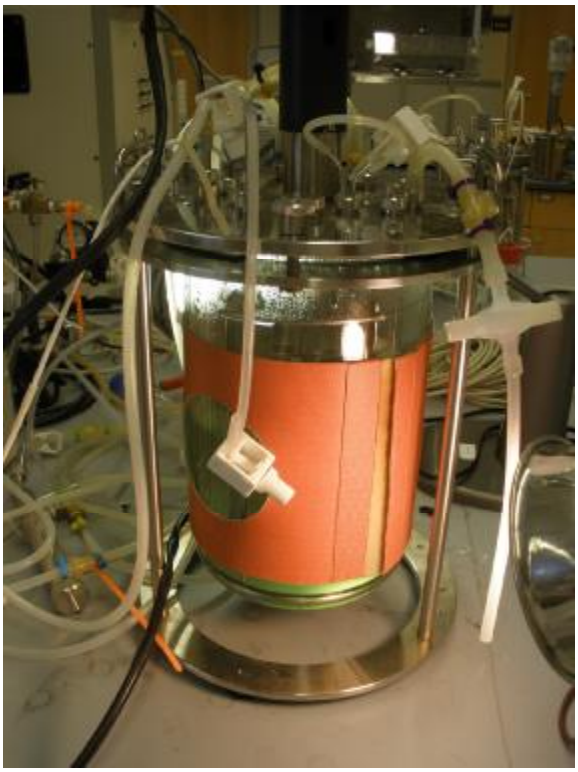


Figure 2.2 ***A. variabilis*** **ATCC 29413 in 5 L fermentor**

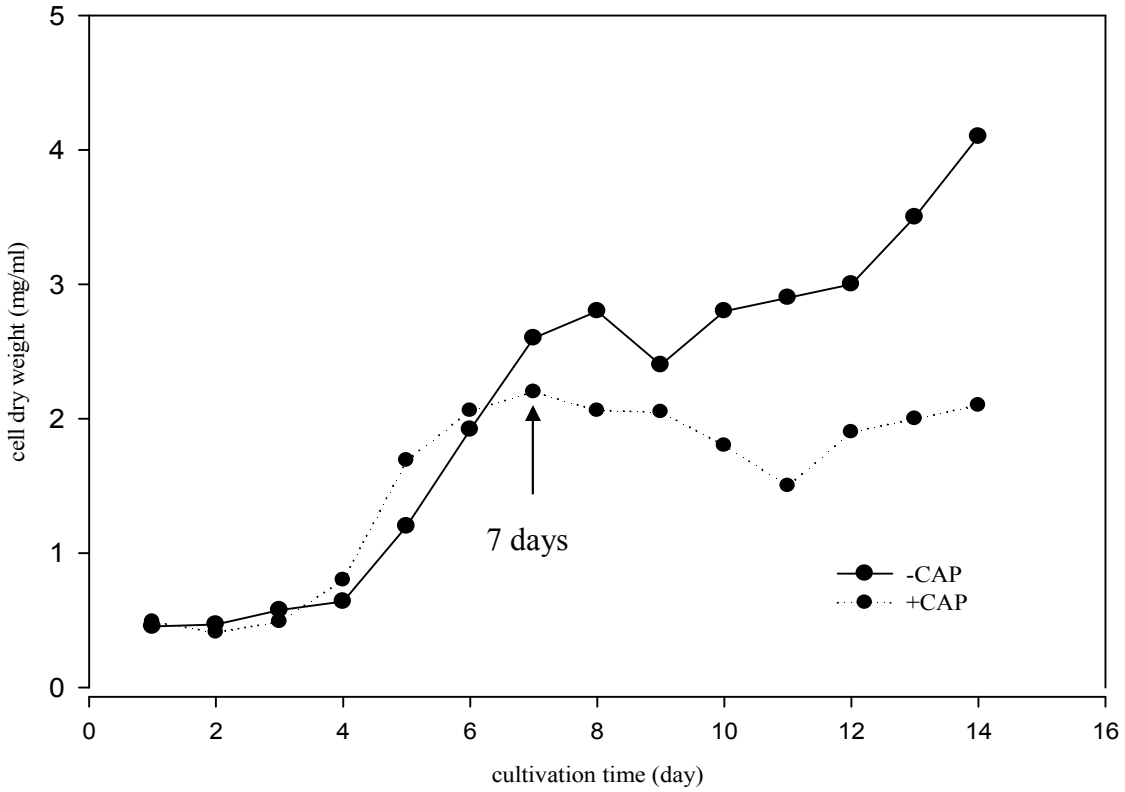


Figure 2.3 Growth profile of *A. variabilis* ATCC 29413

-CAP denoted chloramphenicol was not added to culture

+CAP denoted chloramphenicol was added to culture

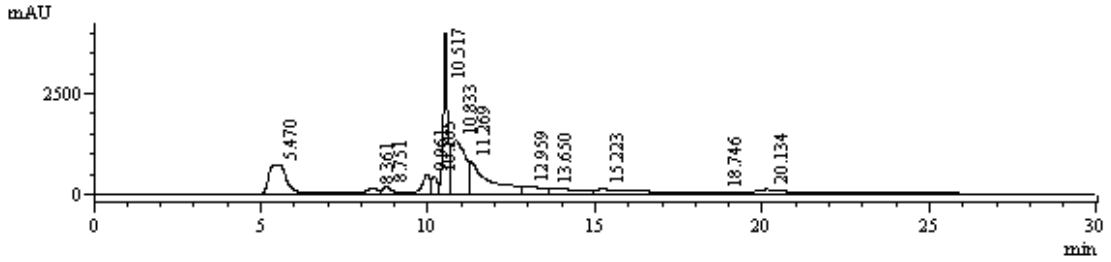


Figure 2.4 HPLC profile of standard protein

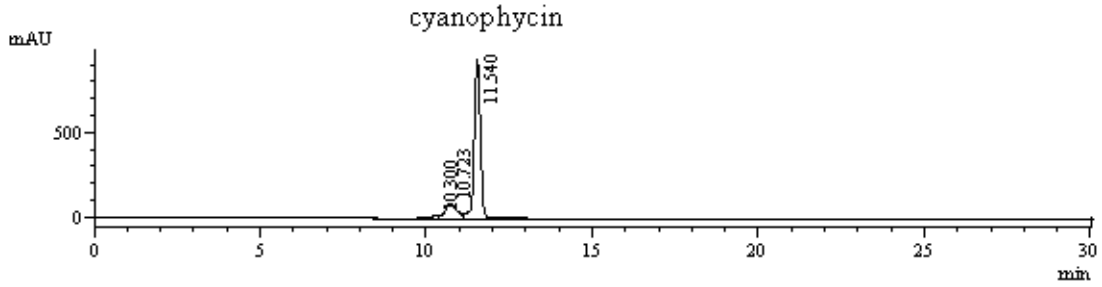


Figure 2.5 HPLC profile of cyanophycin from *A. variabilis* ATCC 29413

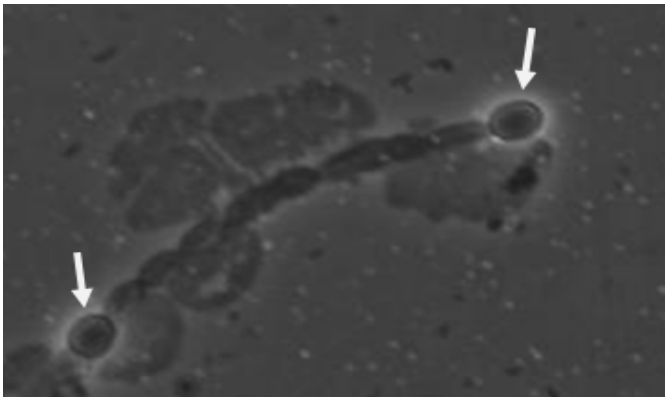


Figure 2.6 Cyanophycin granules in cells of *A. variabilis* ATCC 29413

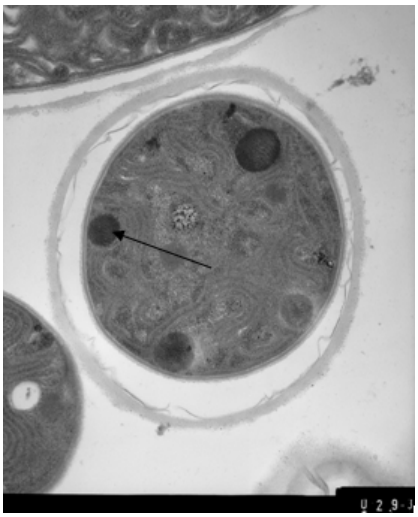


Figure 2.7 Ultrastructure of *A. variabilis* ATCC 29413

CHAPTER 3 - Development of recombinant *E.coli* strain for production of cyanophycin

Introduction

Cyanophycin synthetase

The polymerization reaction of cyanophycin is catalyzed by cyanophycin synthetase (CphA). The molecular weight of it is usually around 100 kDa (Füser and Steinbüchel, 2007). CphA generally requires Asp, Arg, ATP, Mg^{2+}, and a primer (low-molecular mass cyanophycin) for *in vitro* cyanophycin synthesis. It is also reported that a thiol reagent and K^+ enhanced the activity of CphA (Simon, 1976; Ziegler *et al*., 1998). CphA does not synthesize cyanophycin *in vitro* without a primer (Aboulmagd *et al*., 2000). However, a cyanophycin synthetase from *Thermosynechococcus elongatus* BP-1 catalyzes *in vitro* cyanophycin synthesis in the absence of a primer (Arai and Kino, 2008). The energy for peptide bond formation is provided by ATP (Ziegler *et al*., 1998). A primer is elongated at its C-terminus, and Asp and Arg are incorporated in a stepwise manner with Asp followed by Arg (Berg *et al*., 2000). CphA from *Anabaena variabilis* PCC 7937 synthesizes cyanophycin by using $(\beta\text{-Asp-Arg})_3$ as a primer (Berg *et al*., 2000), and CphA from *Synechocystis* sp. PCC 6308 does not use poly-α-L-aspartic acid as a primer (Aboulmagd *et al*., 2001a). The mechanism of primer supply *in vivo* is still unclear; neither the enzyme nor the gene encoding the enzyme that catalyzes primer synthesis has been identified.

Sequence analysis shows that CphA has two catalytic domains, and each domain showed similarity to an ATP-grasp domain or a Mur ligase domain (Murzin, 1996; Füser and

Steinbüchel, 2007). It appears that one catalyzed the ligation of Asp, and the other is responsible for the ligation of Arg.

The *cphA* genes can be expressed in several bacteria and plants. The recombinant culture has the ability to produce comparably large amounts of cyanophycin in a much shorter period of time as compared to cyanobacteria (Frey *et al*., 2002; Elbahloul *et al*., 2005b). The cyanophycin genes (*cphA*) from *Anabaena* sp. strain PCC 7120, *Synechocystis sp. strain* PCC 6803, *Synechocystis* sp. strain PCC 6308, *Synechococcus elongatus* and *Synechococcus* sp strain MA 19 were cloned and expressed in *Escherichia coli* (Hai *et al*., 1999; Oppermann-Sanio *et al*., 1999; Aboulmagd *et al*., 2000; Berg *et al*., 2000). The molecular mass of cyanophycin synthesized by recombinant *E.coli* became much smaller but homogenous, approximately 25 to 30 kDa, and usually contained lysine as an additional amino acid (Ziegler *et al*., 1998; Hai *et al*., 2000; Berg *et al*., 2000; Aboulmagd *et al*., 2001b; Krehenbrink *et al*., 2002).

pET system and protein detection

Due to the wide knowledge of metabolism and available genetic tools of *Escherichia coli*, it becomes the most commonly used bacterial hosts for the production of recombinant proteins (Lee. 1996). Several expression systems have been developed for technical-scale production of recombinant protein in *E.coli* based on the regulated trp, lac, or lambda PL promoter (Blaber, 1998.)

One of the most powerful systems developed so far for the cloning and expression of recombinant proteins in *E.coli* is pET system. Target genes are cloned in pET plasmids under control of strong bacteriophage T7 transcription and translation signals. Expression is induced by providing a source of T7 RNA polymerase in the host cell. There are two ways to accomplish this goal: one is to introduce bacteriophage into the host cell, for example, λCE6. The other way

is to transfer the plasmid into an expression host containing a chromosomal copy of the T7 RNA polymerase gene under lacUV5 control. In the second case, expression is induced by the addition of IPTG or lactose to the bacterial culture. T7 RNA polymerase is so selective and active that when fully induced, almost all of the cell's resources are converted to target gene expression. The desired product can occupy more than 50% of the total protein a few hours after induction.

The vector used in this study is pET45b+. It contains several important elements (Figure 3.1): a lacI gene, which codes for the lac repressor protein; a T7 promoter which is specific to T7 RNA polymerase; a lac operator which can block transcription; multiple cloning sites where our target gene can be inserted; a f1 origin of replication; an ampicillin resistant gene; and his-tag coding sequence which encodes six histidine and binds to our target protein (Blaber, 1998).

To start the process, our gene of interest (GOI) needs to be cut by restriction enzyme and inserted into the multiple cloning site of pET45b+ vector. Both T7 promoter and the lac operator are located at 5' to GOI. When the T7 RNA polymerase is present and the lac operator is not repressed, the transcription of GOI proceeds rapidly.

The host cells used in this study is *E.coli* strains BL21 (DE3) pLysS and BL21 (DE3). They have been genetically engineered to incorporate the gene for T7 RNA polymerase, the lac promoter and the lac operator in their genomes. When lactose or lactose analogue, for example isopropyl β-D-1-thiogalactopyranoside (IPTG), is present inside the cell, transcription of T7 RNA polymerase is activated (Blaber, 1998). The difference between BL21 (DE3) pLysS and BL21 (DE3) is that BL21 (DE3) pLysS contains pLysS plasmid (Figure 3.2). The pLysS plasmid is constructed by insertion of T7 lysozyme gene, which encodes T7 lysozyme: a natural inhibitor of T7 RNA polymerase to suppress basal expression, and pLysS plasmids contain

chloramphenicol resistant encoding sequence, which makes BL21 (DE3) pLysS strain resistant to chloramphenicol. Figure 3.3 is the overview of the pET system process.

Target protein is detected by SDS-PAGE analysis followed by staining with Coomassie blue, which in many cases will reveal the target protein as a unique band when run adjacent to an uninduced negative control sample. Western blot is a more specific and sensitive method for identification of expression, and can be conveniently performed using fusion tag-specific reagents or protein-specific antibodies or other ligands. Figure 3.4 shows brief procedure of western blot. The whole *E.coli* cells are taken as sample and denatured in sample buffer and protein in the sample are separated using SDS-PAGE electrophoresis. In order to make the protein accessible to antibody detection, they are moved from within the gel onto a membrane of nitrocellulose or polyvinylidene difluoride (PVDF). The membrane is placed on top of the gel, and a stack of filter paper placed on top of that. The entire stack is placed into an electrophoresis cassette. Electric current is used to pull the proteins from the gel onto the membrane while maintaining the organization they had within the gel. The membrane is designed to bind protein and both antibody and target protein can be bound to the membrane. Blocking buffer which is a diluted solution of protein typically BSA or non-fat dry milk is applied to attach to the membrane in all places where the target proteins have not attached. Thus when the antibody is added, there is no room on the membrane for it to attach other than on the binding sites of the specific target protein. This reduces the noise background in the final product of the western blot, leading to clearer results and eliminates false positives. The next step is detection. The membrane is probed for the protein of interest with antibodies. Usually two antibodies are used, first is known as primary antibody, which directly attaches to the protein of interest. In this case, our target protein is linked with six histidines, so the first antibody used is anti-his which can bind to the histidine

tag. After rinsing the membrane to remove unbound primary antibody, the membrane is exposed to secondary antibody, directed at a species-specific portion of the primary antibody. In this case, the primary antibody is a mouse-source antibody, so the secondary antibody is anti-mouse that can bind to almost any mouse-sourced primary antibody. The secondary antibody is usually linked to a reporter enzyme such as alkaline phosphatase. When the membrane is rinsed in a substrate that reacts with the reporter enzyme, the soluble dye is converted into insoluble form of a color that precipitates next to the enzyme and thereby stains the membrane. Color development of the blot is then stopped by washing away the soluble dye. The intensity of the band is proportional to the amount of protein expressed.

Till date, cyanophycin could only be produced at the laboratory scale for analytical purpose. The aim of this study was to establish an efficient recombinant strain for the large scale production of cyanophycin. Therefore, the objective is to clone cyanophycin synthetase gene from *A. variabilis* ATCC 29413, introduce *cphA* gene into *E.coli* cells and express cyanophycin synthetase.

Materials and methods

Strains and plasmids

Strains and plasmids used in this study are listed in Table 3.1. *Escherichia coli* strains BL21 (DE3) pLysS and BL21 (DE3) purchased from EMD Biosciences (USA) were used as host cells for construction of recombinant strain in this study. BL21 (DE3) pLysS contains a pLysS plasmid which can encode T7 lysozme for a stringent control over the protein expression. pET45b+ was used for cloning and expression vector for *cphA*. It is a high copy plasmid, which had multiple cloning sites and his-tag sequence at its N-terminal.

Culture growth conditions

BL21 (DE3) pLysS and BL21 (DE3) were grown at 37 ºC in Luria Bertani medium containing 10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl, ampicillin (50 µg/ml) and chloramphenicol (5 µg/ml), or ampicillin (50 µg/ml) only, respectively. Growth was monitored by measuring the turbidity at 600 nm. *A. variabilis* ATCC 29413 was grown at 30 ºC in BG-11 medium according to the instruction of ATCC.

Isolation of genomic DNA from A. variabilis ATCC 29413

Total genomic of DNA from *A. variabilis* ATCC 29413 was prepared according to the procedure described by Narayanan *et al.,* (2001). Briefly, *Anabaena* cells were pelleted by centrifugation at 5000×g for 10 min at 4 ºC. The pelleted cells were washed and resuspended in 10 ml TES buffer (25% sucrose, 50 mM Tris [pH 7.5], and 1 mM EDTA,) and transferred to 50 ml centrifuge tube. The cells were centrifuged for 10 min at 8000×g. The cells were then treated with lysozyme (1 mg/ml) for 30 min and lysed with sarkosyl (1%) followed by RNaseA (20 µg/ml) and incubated at 37 ºC for 30 min and cooled on ice. The prepared samples were further sequentially extracted with equal volume of buffer-saturated phenol and chloroform. DNA from the extract was then precipitated in 1/10 volume of sodium acetate (3 M, pH 5.2) and 0.7 volume of isopropanol (2.5 volume of 75% ethanol), resuspended in TE (10 mM Tris-HCl [pH 8.0], and 1 mM EDTA) and stored at 4 ºC until used.

Primer design and PCR

Primers for PCR amplification of *cphA* from *A. variabilis* ATCC 29413 was designed based on the sequence (GenBank NC-007413.1) deposited in NBCI database. Forward primer (5'-TCCGAGCTCATGAGAATCCTCAAGATCCAG-3') contained a SacI at the 5' end while

the reverse primer (5'-AAACTGCAGCTACAGCAAAGTATTAATAATTACAGA-3') carried a PstI site in the 5' end. Primers were obtained from Integrated DNA Technologies, Coralville, IA, USA.

PCR amplifications were carried out under the following conditions using ExTaq (TAKARA BIO Inc, Madison, WI, USA): 1 cycle at 94 ºC for 3 min followed by 35 cycles of denaturation at 9 ºC for 1 minute, annealing at Tm 45 ºC and 49 ºC each for 30 seconds, and extension at 72 ºC for 2.5 min. The amplified products were subjected to electrophoresis in 1% agarose gel, purified with Wizard SV gel and PCR clean up system (Promega Corp. Madison, WI, USA).

Recombinant vector preparation

cphA PCR products and pET45b+ vector were digested with SacI and PstI restriction enzymes. The digestion formulas are presented in Tables 3.2 and 3.3, respectively. Digestion mixture was incubated at 37 ºC water bath for 4 hours, and then ligated at 16 ºC with T4 DNA ligase to form recombinant pET45b+ vector.

Transformation of recombinant pET45b+ vector into BL21 (DE3) pLysS

2 µl ligation products (pET45b+ vector with *cphA* gene) were added to 20 µl BL21 (DE3) pLysS competent cells. Cells were then incubated on ice for 5 min and heated for exactly 30 seconds in 42 ºC water bath followed by cooling on ice for 2 min. 80 µl SOC medium was added and cells were incubated at 37 ºC while shaking at 250 RPM for 60 min prior to plating on selective media. The colonies that appeared on the plate were selected and the cloned pET45b+ plasmid were isolated using AurumTM plasmid mini kit (Bio-Rad, CA, USA), The isolated plasmids were sent for sequencing to the Institute for Integrative Genome Biology,

University of California, Riverside. The sequence thus obtained was verified for in-frame cloning using Vector NTI software.

Transformation of recombinant pET45b+vector into BL21 (DE3)

Plasmids were extracted from recombinant BL21 (DE3) pLysS by AurumTM plasmid mini kit (Bio-Rad, CA, USA), digested with EcoRI to remove pLysS plasmid. The digested plasmids were subjected to electrophoresis in 1% agarose gel, purified with Wizard SV gel and PCR clean up system, and then transferred into BL21 (DE3) competent cells.

Analysis of transformants

Ligation screening

To verify ligation between vector and insert, a ligation reaction was analyzed directly by PCR using insert-specific primers, which were the same set of primers used to amplify the insert (*cphA*) and the same PCR procedure described in Primer design and PCR conditions.

Restriction enzymes analysis of recombinant pET45b+ vector

Plasmids were extracted from colonies by using AurumTM plasmid mini kit (Bio-Rad, CA, USA) and analyzed by double digestion with SacI and PstI and single digestion with HindIII. Digestion reaction formulas are presented in Tables 3.3 and 3.4, respectively.

Colony PCR

Recombinant BL21 (DE3) colonies were screened for inserts by doing colony PCR according to the following procedure. Single colony was picked and boiled in 50 µl sterile water for 5 min to lyse the cells and denature DNases, centrifuged at 10,000×g (Expendorf centrifuge

5415R, Hamburg, Germany) for 1 min to remove debris and 10 µl supernatant containing DNA was used for PCR analysis. PCR program was described in Primer design and PCR conditions.

Induction of target protein

Single colony was picked from a freshly streaked plate and inoculated into 5 ml LB broth containing antibiotics and cultivated at 37 ºC overnight as starter culture. 5 ml of this starter culture was then inoculated into 50 ml fresh LB broth with the same antibiotics and grown at 37 ºC until the OD_{600} was approximately 0.4. 0.5 M IPTG was added to final concentration of 2 mM and the incubation was continued at 37 ºC for 4 hours.

Detection of target protein

SDS-PAGE electrophoresis

Whole cells were separated in 11% (wt/vol) sodium dodecyl sulfate-polyacrylamide gel as described by Laemmli (1970). Gel formulation is shown in Table 3.5. Separating gel was firstly poured into gel caster, left about 2 centimeters below the bottom of the comb for the stacking gel. After the separating gel solidified; stacking gel was poured above it, comb was inserted to make wells for loading samples. Cells were mixed with sample loading buffer and boiled for 5 minutes before loading. Gel was run at 130 V constant for one hour (mini electrophoresis system, BIO-RAD, Hercules, CA, USA). Proteins were stained with Coomassie brilliant blue G-250. Kaleidoscope Prestained Standards were used (BIO-RAD, Hercules, CA, USA).

Western blot

Proteins in the unstained SDS gel were electro-transferred onto nitrocellulose membrane (Millipore Corp., MA, USA) using Biorad mini trans-blot module (Biorad, Hercules, CA, USA).

Membranes were blocked with 3% BSA buffer, anti-his was used to detect the proteins, anti-mouse was used as the secondary antibody, and the immunoreactive proteins were detected using 5-bromo-4-chloro-3-indolyphosphate/nitro blue tetrazolium (BCIP/NBT) as substrate.

Results and discussion

Cloning of cyanophycin synthetase gene

A 2.7 kb PCR product corresponding to the size of *cphA* gene (GenBank NC-007413) from *A. variabilis* ATCC 29413 was successfully amplified (Figure 3.5). A band located between 2.5 and 3 kb can be seen clearly.

Analysis of BL21 (DE3) pLysS transformant

PCR result (Figure 3.6) showed that the *cphA* gene was successfully inserted into pET45b+ vector. The intense 2.7 band was *cphA* gene that was amplified by using the ligation mixture as template.

Plasmids from recombinant BL21 (DE3) pLysS colonies 1 to 13 was digested with HindIII (Figure 3.7). After digestion, plasmids from colony 1, 2, 5, 6, 7, 8, 9, 11, 12, and 13 resulted in two DNA fragments, one was around 8kb and the other was 4.9 kb corresponding to the size of recombinant pET45b+ vector and pLysS plasmid, respectively. Colonies 2, 3, 4 and 10 were excluded because they did not yield the 8 kb band, which suggested that the recombinant pET45b+ vector was not transferred into *E.coli* cells.

Plasmids from the remaining colonies were further analyzed by double digestion with SacI and PstI. Figure 3.8 showed the results. Plasmids from colony 6, 7, 8, 9, 11, 12 and 13 released a 2.7 kb band (*cphA*), which were considered to be successful transformants and were induced by IPTG to express cyanophycin synthetase.

DNA sequencing of recombinant pET45b+ vector was performed by Institute for Integrative Genome Biology, University of California, Riverside. The order of the nucleotide bases of the PCR product was determined, and the results matched with the nucleotide bases order of cyanophycin synthetase gene from *A. variabilis* ATCC 29413. **Therefore, DNA sequencing for the recombinant pET45b+ finally proved that *cphA* gene was successfully cloned into pET45b+ vector.**

Analysis of BL21 (DE3) transformant

Colony PCR and HindIII digestion results (Figure 3.9 and 3.10) proved that the recombinant pET45b+ vector was transformed into BL21 (DE3) successfully. *cphA* gene was amplified successfully after colony PCR. There were some bands showing at the 1 kb position; these bands may be some DNA fragment amplified from region of chromosome DNA of *E.coli*, which has the complimentary sequence of primers we used. HindIII digestion linearized recombinant pET45b+ vector, resulting in an 8 kb band.

Expression of cphA in recombinant BL21 (DE3) pLysS

The expression of cyanophycin synthetase from recombinant BL21 (DE3) pLysS strain was monitored after inducing the culture with IPTG at 0.4 OD_{600} value. The measurement of the Optical Density (OD) value was performed using multiprocessor-based UV-Vis Spectrophotometer. After IPTG induction, the OD_{600} value of recombinant BL21 (DE3) pLysS slightly decreased when compared with the OD_{600} value of BL21 (DE3) pLysS control, which suggested that expression of *cphA* slowed the growth of recombinant *E.coli*.

Expression of *cphA* occurred in recombinant *E.coli* in the presence of IPTG. Molecular weight of cyanophycin synthetase produced by recombinant *E.coli* was around 100 kDa (Figure

3.11) corresponding to the size of cyanophycin synthetase from *A. variabilis* ATCC 29413, which was 98 kDa (Füser *et al*., 2007).

Proteins were further detected by western blot (Figure 3.12). After color development, there were bands located at 100 kDa that corresponded to colonies 6, 7 and 13, respectively. Only colony 13, 7, and 6 was capable to express cyanophycin synthetase. Based on the band intensity, we decided to focus on colony 7 for further analysis. Reducing IPTG induction time resulted in less protein expression (Figure 3.13).

Expression of cphA in recombinant BL21 (DE3)

Recombinant BL21 (DE3) expressed much more CphA than recombinant BL21 (DE3) pLysS (Figure 3.14). Cyanophycin synthetase from BL21 (DE3) pLysS was detected as a sharp and light band, while Cyanophycin synthetase from BL21 (DE3) was detected as dark multiple bands, this may due to too much protein expression.

Conclusions

pET expression system was selected to produce cyanophycin synthetase. The pET45b+ vector was used and the expression of cyanophycin synthetase was induced by IPTG. *cphA* gene from *A. variabilis* ATCC 29413 was successfully cloned into the BL21 (DE3) pLysS and BL21 (DE3), which was proven by plasmid analysis, colony PCR, and DNA sequencing. Expression of *cphA* in these recombinant *E.coli* cells was successful; both of them produced the cyanophycin synthetase. Cyanophycin synthetase was produced as a C-terminal his-tagged protein, and western blot result indicated a molecular weight of it about 98kDa. Without the control of pLysS plasmids, T7 lysozme was not synthesized, the level of T7 RNA polymerase was high, and transcription of *cphA* proceeded rapidly, therefore leading to more cyanophycin synthetase

production in recombinant BL21 (DE3) than recombinant BL21 (DE3) pLysS. Western blot results showed that cyanophycin synthetase from BL21 (DE3) was detected as multiple dark bands, while cyanophycin synthetase from BL21 (DE3) pLysS was detected as a light and sharp band. The dark multiple bands may be due to excess protein expression. IPTG induction time and concentration were critical for cyanophycin synthetase formation. Better enzyme production was obtained at IPTG final concentration of 2 mM and 4 hours induction time. The functionality of cyanophycin synthetase in these recombinant *E.coli* cells needs to be further proved. The next objective is to produce cyanophycin *in vivo* using these recombinant *E.coli* strains.

Table 3.1 Strains and plasmids used

E.coli strains	Relevant characteristics	Source
BL21 (DE3) pLysS	*E.coli* B F- dcm omp T hsdS(rB- mB-) gal λ (DE3) [pLysS Cam]	EMD Biosciences, USA
Plasmids		
pET45b+	Apr T7 promoter his Tag coding sequence	Novagen (Rockland, MA)
pET45b+::*cphA*	pET45b+ carrying 2.7kb PCR product from *Anabaena variabilis* ATCC 29413 genomic DNA harboring *cphA*	This study

Table 3.2 Formula of insert restriction digestion with SacI and PstI

Name	Volume (µl)
Water	0
Buffer 1	3
BSA	0.3
Insert DNA (*cphA*)	25
SacI	1
PstI	1
Total volume	30

Table 3.3 Formula of pET45b+ / recombinant pET45b+ vector digestion with SacI and PstI

Name	Volume (µl)
Water	9.2
Buffer 1	2
BSA	0.2
pET45b+ /pET45b+ recombinant	7
SacI	0.8
PstI	0.8
Total volume	20

Table 3.4 Formula of recombinant pET45b+ vector digested with HindIII

Name	Volume (µl)
Water	10.2
Buffer 2	2
HindIII	0.8
pET45b+ recombinant plasmid	7
Total volume	20

Table 3.5 SDS-PAGE formula

	Double distiller water (ml)	40% degassed Acrylamid/bis (ml)	Gel buffer (ml)	10% w/v SDS (ml)
Separating gel	4.625	2.775	2.5	0.1
Stacking gel	6.425	0.975	2.5	0.1

For 10 ml volume

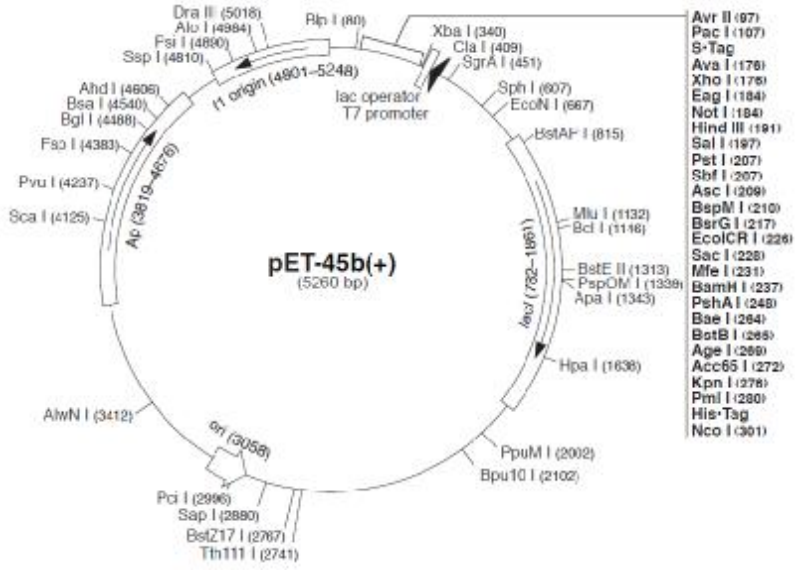


Figure 3.1 pET45b+ vector map (Novagen)

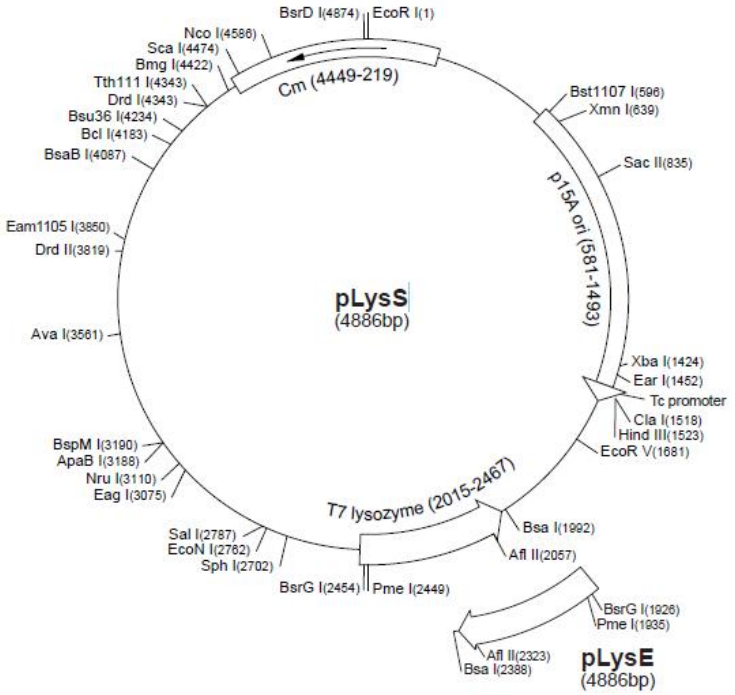


Figure 3.2 pLysS plasmid map (Novagen)

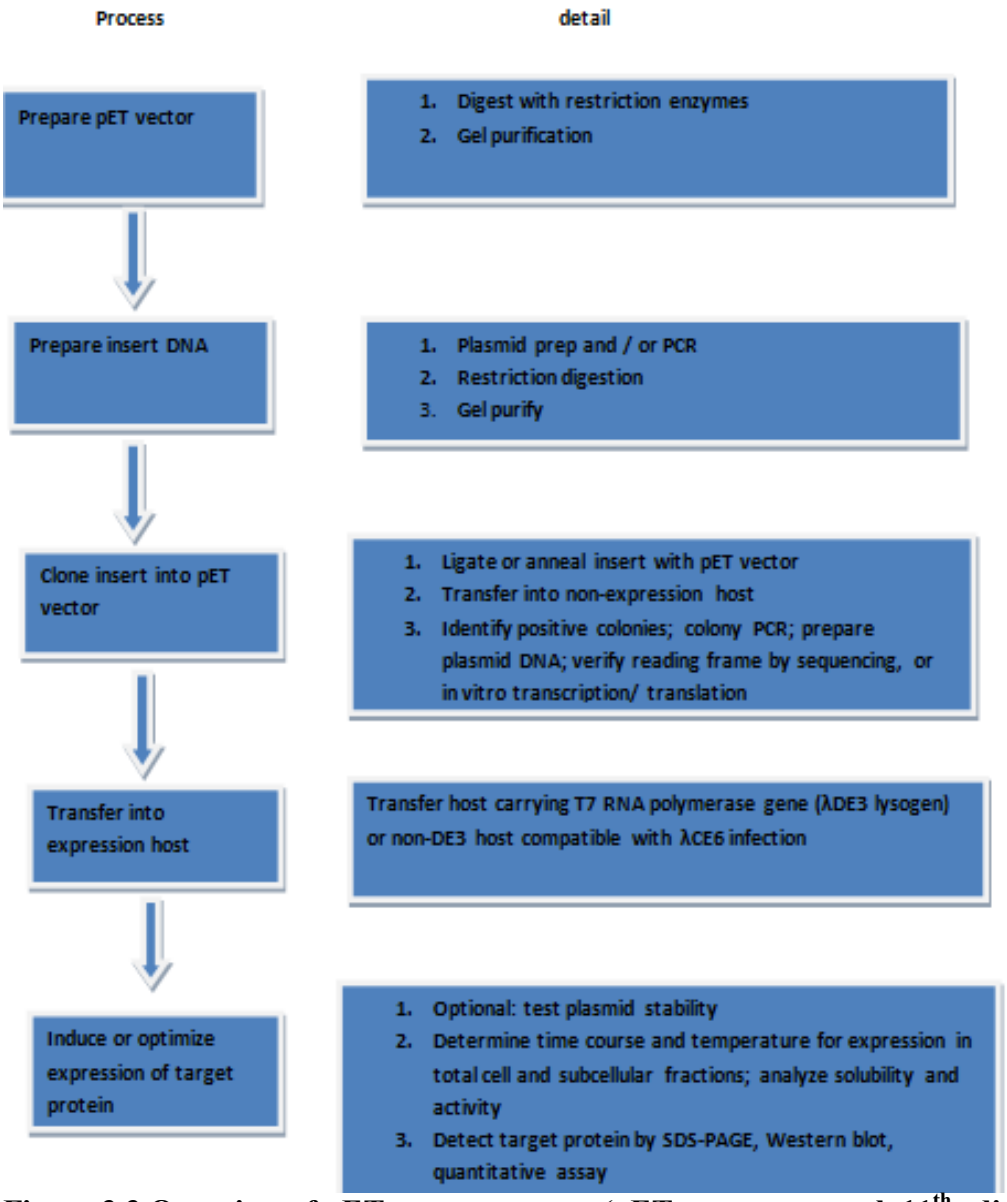


Figure 3.3 Overview of pET system process (pET system manual, 11th edition, Novagen)

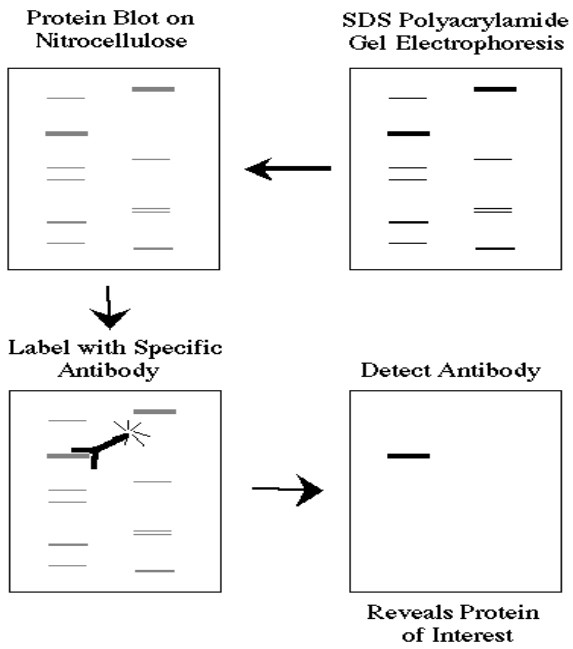


Figure 3.4 Brief overview of western blot
(http://www.bio.davidson.edu/courses/genomics/method/Westernblot.html)

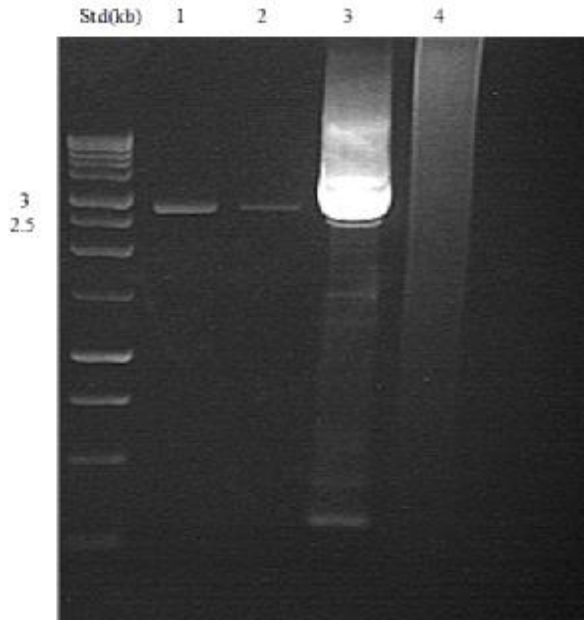


Figure 3.5 PCR products of cyanophycin synthetase gene
Lane 1-2: PCR product (*cphA*); lane 3: positive control; lane 4: negative control

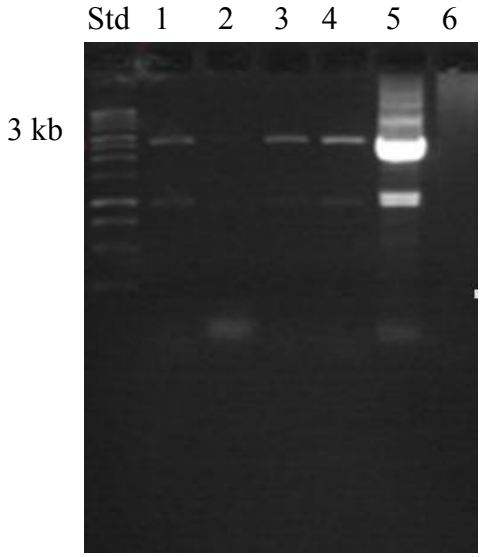


Figure 3.6 Ligation analysis
Lane:1-4: PCR product (*cphA* gene) using chromosomal DNA of *A. variabilis* ATCC 29413 as template; lane 5: product (*cphA* gene) using ligation mixture (recombinant pET45b+ vector with cphA gene) as template; lane 6: negative control (water instead of template)

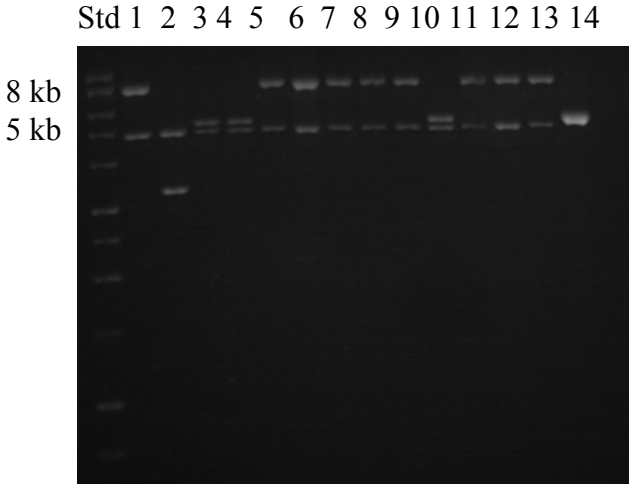


Figure 3.7 plasmids extracted from colony 1 to 13 digested with Hind III
Lane 1-13: plasmids digested with HindIII from cololny 1-13, respectively; lane 14: pET45b+ plasmid digested with HindIII

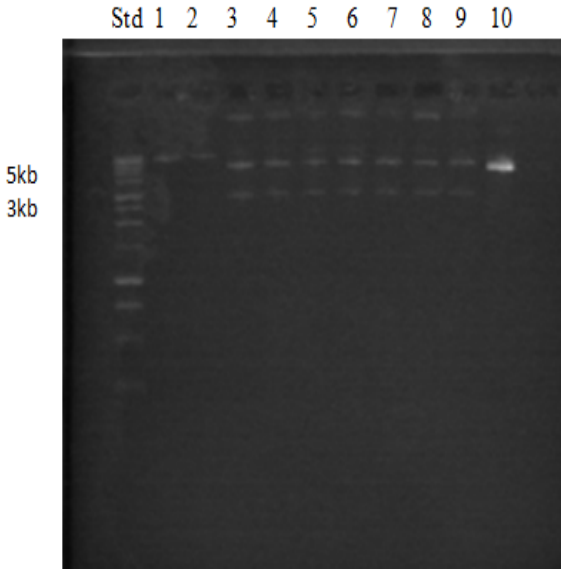


Figure 3.8 Plasmids digested with SacI and PstI
Lane1-9: Plasmids from colony 1, 5-9, and 11-13 digested with SacI and PstI respectively; lane 10: pET45b+ vector digested with SacI and PstI

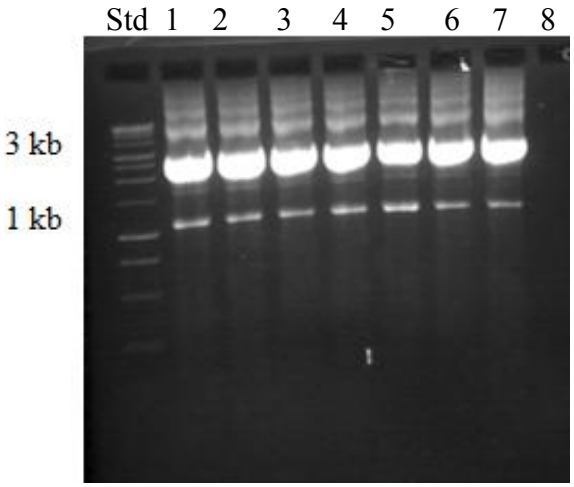


Figure 3.9 Colony PCR
Lane 1-6: PCR product using DNA from recombinant BL21 (DE3) (colony 1-6) as template; lane 7: positive control; lane 8: negative control

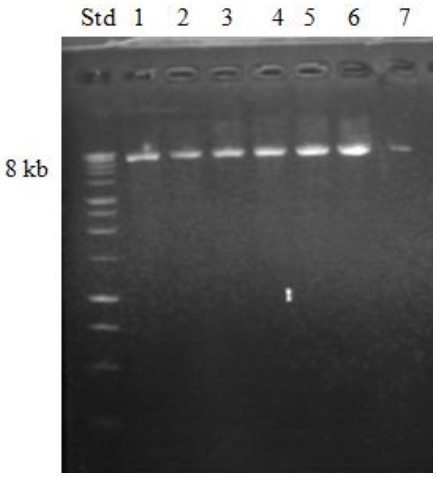


Figure 3.10 Plasmids extracted from recombinant BL21 (DE3) digested with HindIII
Lane 1-6: plasmids extracted from colony 1-6 digested with HindIII, respectively; lane7: pET45b+ vector digested with HindIII

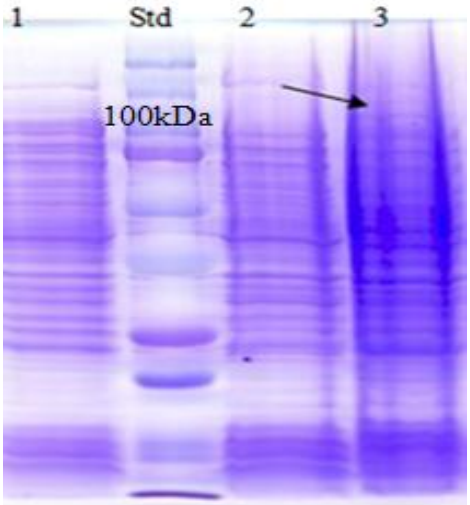


Figure 3.11 SDS-PAGE analysis of recombinant cyanophycin synthetase (CphA)
Lane 1: IPTG induced BL21 (DE3) pLysS containing pET45b+ without *cphA*; lane 2: IPTG uninduced recombinant BL21 (DE3) pLysS; lane 3, IPTG induced recombinant BL21 (DE3) pLysS

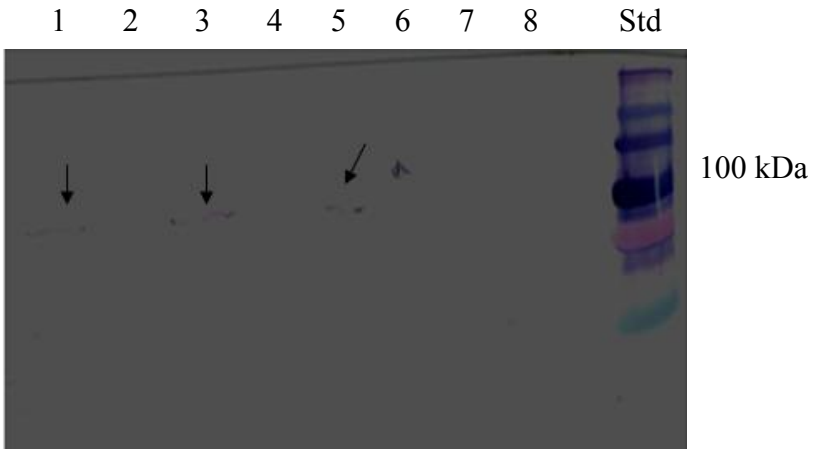


Figure 3.12 Western blot for detecting cyanophycin synthetase (CphA)
Lane 1, 3, 5, and 7: IPTG induced recombinant BL21 (DE3) pLysS colony 13, 7, 6 and 12; lane 2,4, 6 and 8: IPTG uninduced recombinant BL21 (DE3) pLysS colony 13,7,6 and 12

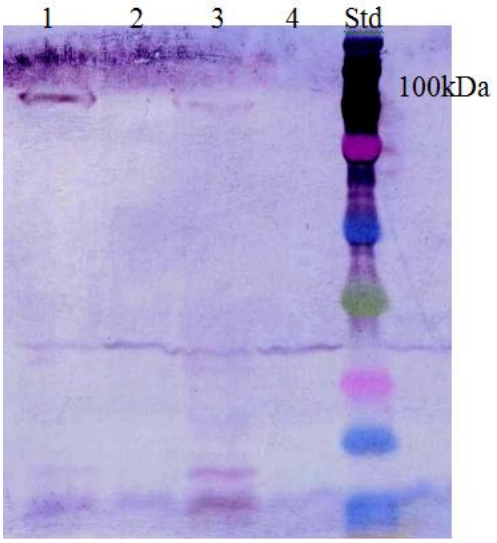


Figure 3.13 Effect of induction time on protein expression
Lane 1: IPTG induced recombinant BL21 (DE3) pLysS (4 hours induction); lane 2 and 4: IPTG uninduced recombinant BL21 (DE3) pLysS*;* lane 3: IPTG induced recombinant BL21 (DE3) pLysS (3hours induction)

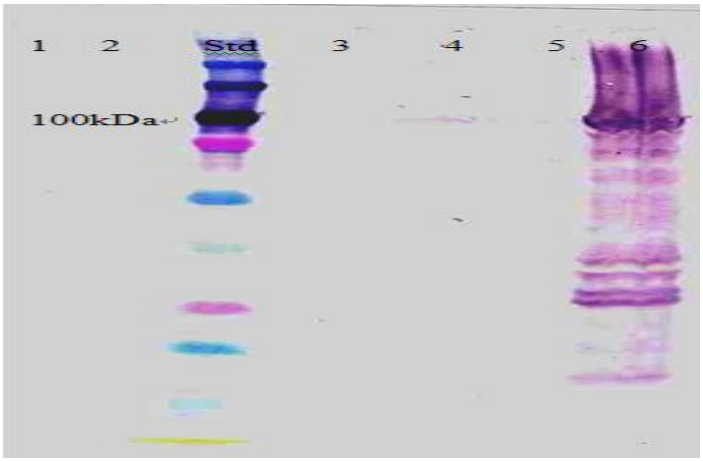


Figure 3.14 Western blot of detecting cyanophycin synthetase produced by recombinant BL21 (DE3) pLysS and BL21 (DE3)
Lane 1: IPTG induced BL21 (DE3) pLysS containing pET45b+ without *cphA*; lane 2, IPTG induced BL21 (DE3) containing pET45b+ without *cphA*; lane 3: IPTG uninduced recombinant BL21 (DE3) pLysS; lane 4: IPTG induced recombinant BL21 (DE3) pLysS; lane 5: IPTG uninduced recombinant BL21 (DE3); lane 6: IPTG induced recombinant BL21 (DE3)

CHAPTER 4 - Characterization and production of cyanophycin

Introduction

Chemical properties of cyanophycin

Cyanophycin is a nitrogen-rich compound which is also known as cyanophycin granule polypeptide or multi-arginyl-poly-L-aspartic acid. It consists of equal-molar quantity of aspartic acid and arginine, aspartic acid arranged as a poly backbone, with arginine residue linked to the β-carboxyl groups via isopeptide bonds (Simon and Weathers 1976). Because of the isopeptide bonds, cyanophycin is resistant to proteolytic enzymes and requires special hydrolytic enzymes for degradation.

According to its chemical structure, cyanophycin is a zwitterionic molecule with a predicted isoelectric point of about 7. Simon and Weathers (1976) did polyacrylamide gel isoelectric focusing. A main band with IP=4.75 and a minor band with IP=6.1 was observed for cyanophycin from *Anabaena cylindrica*. The ratio of aspartic acid to arginine is 1:1 for the IP=6.1 band, while the main band at pH 4.75 may have lost some of its arginyl residues.

Cyanophycin is insoluble at neutral pH. Simon *et al*. (1980) attempted to get information on the secondary structure of cyanophycin by circular dichroism and Laser Raman spectroscopy. The results suggested about 50% β sheets, 45% random coil and 0-5% α helix as secondary structure. These spectral features were sensitive to the environment; they were destroyed at alkaline pH as well as high concentrations of urea. These spectra were taken as evidence that the insoluble form of cyanophycin has “some” β sheet and no α helix as secondary structure. The structure of cyanophycin under physiological conditions is β sheet. In this case, the “side chain” of adjacent amino acids of the poly-α-aspartic acid backbone, which in the branched polypeptide

cyanophycin include the arginyl groups, would point in opposite directions. Because of the large size of the "side chain", the arginyl residues of one strand may slip in between arginyl residues of another strand, resulting in stacking of strands or sheets. In such a structure, the positive charges on the guanidino groups (CN3H4) of one strand would interact electrostatically with the negative charges on the α-carboxyl groups of the arginine moieties of the other strand (Simon *et al*., 1980). In this way, many cyanophycin molecules could form a large, insoluble complex. Acidic pH and high ionic strength would dissolve the polymer because they diminish the Coulomb interactions. High concentrations of urea and the strong detergent sodium dodecyl sulfate could also dissolve the polymer because they destroy the secondary structure.

Characterization of cyanophycin

Characterization of cyanophycin was a big challenge for us, because standard cyanophycin is not commercially available and cyanophycin is very polydispersed. Quantification of cyanophycin can be done in several ways. Allen *et al.* (1980) had determined the number and size of cyanophycin granules using light microscopy and electron microscopy. The amount of polymer can also be determined by amino acid analysis after complete hydrolysis with HCl (Aboulmagd *et al*., 2000). This method provided qualitative as well as quantitative information about cyanophycin, but it requires specific equipment and is time-consuming. Erickon *et al*. (2001) developed a sensitive method for quantification of cyanophycin by NMR spectroscopy. HCl extracts of pellets consisting of broken cyanobacterial cells were found to contain cyanophycin sufficiently pure for NMR analysis. The polymer was quantified by integration of the NMR peak representing the protons attached to the δ- carbon atoms of its arginyl moieties; calibration of the peak area with purified cyanophycin is required. Cyanophycin has been determined from rather crude samples by high pressure liquid

chromatography on reverse phase columns, developed with a linear gradient of acetonitrile in water containing 0.1% trifluoroacetic acid (Newton and Tyler, 1987). It was detected at 210 nm since cyanophycin does not contain aromatic amino acids. Although the molecular mass of the cyanobacteria polymer ranges over 25-100 kDa, it was found to elute from the column in one sharp peak. In our work, we performed SDS-PAGE, HPLC, Mass spectroscopy, and amino acid analysis to characterize the polymer produced from recombinant *E.coli* cells.

Production of cyanophycin

Large-scale production of cyanophycin has not been achieved. The high cost of culturing the light-dependent cyanobacteria in photobioreactors is an obstacle for producing cyanophycin. At present, a system based on the heterologous expression of cyanophycin synthetases in bacteria and in plant has been developed (Neumann *et al*., 2005; Arai *et al*., 2008).

Aboulmagd *et al*. (2000) observed the production of large amount of the polymer in recombinant cells of *E.coli* expressing gene *cphA* from *Synechocystis* sp. PCC 6308. A maximum polymer content of 24% of dry weight was determined. However, such high content was only reached in complex culture media which was expensive. Frey *et al*. (2002) could produce cyanophycin on a large scale (300 and 500 liter culture volume), by expressing gene *cphA* from *Synechocystis* sp. PCC 6803 under control of λPL promoter and the temperature sensitive cI187 repressor in *E.coli* DH1. Maximum cyanophycin content between 21% and 24% on a dry weight basis was achieved under optimized conditions, only if 1% casamino acids was added to the mineral salts medium.

In our study, we investigated suitable agricultural byproduct to substitute the expensive mineral medium for production of cyanophcyin. First we evaluated LB broth with addition of casamino acids. Later, we evaluated thin stillage made in bioprocessing lab. The whole stillage

provided by Abengoa. Bioenergy (York, NE) was centrifuged and the supernatant was used for production of cyanophycin. The potato waste which was obtained from Derby Dining hall (Kansas State University), potato was prepared by boiling in water and the leftover solution was used to produce cyanophycin.

The objectives of this study are: to characterize cyanophycin produced by recombinant *E.coli*; develop fermentation process for cyanophycin production; and evaluate potential agricultural waste for cyanophycin production.

Materials and methods

Shake flask level experiments

A culture of recombinant BL (DE3) pLysS or BL21 (DE3) was grown by inoculating the colony in to 5ml LB media containing ampicillin (50 µg/ml) and chloramphenicol (5 µg/ml) or only ampicillin (50 µg/ml) overnight. The overnight grown culture were further used to inoculate 100 ml LB medium containing 1% casamino acids or 5% thin stillage or 1% potato waste with antibiotics as mentioned above. The culture was grown in an orbital shaker at 37 ºC and 160 RPM until the OD_{600} value reached 0.4. 0.5 M IPTG was added to the final concentration of 2 mM. Cultivation was continued at 37 ºC for next 10 hours. Cells were harvested and cyanophycin was obtained using acid extraction method.

Fermentor experiments

Fermentation inoculum preparation

12 h 5 ml recombinant BL21 (DE3) pLysS grown for 12 hours was used to inoculate 100 ml LB broth with antibiotics, incubated for 12 h under aerobic conditions at 37 ºC, 160 RPM

(Innova 4000, Incubator Shaker, New Brunswick Scientific, USA), and transferred into pre-sterilized centrifuge bottles and centrifuged at 3000×g for 20 min (Model TJ-6 Centrifuge, Beckman Instruments, USA). After centrifugation, the supernatant was discarded and the cell pellet was re-suspended with 100 ml of fermentation medium (described in the fermentation medium and conditions section) into a sterilized empty flask and used as inoculum for the batch fermentation experiment.

Fermentation medium and conditions

The fermentation medium consisted: glucose (20 g/l), tryptone (10 g/l), yeast extract (5 g/l), NaCl (10 g/l), antibiotic (ampicillin 50 µg/ml, chloramphenicol 5 µg/ml) and casamino acids (10 g/l). Casamino acids, glucose and LB broth were sterilized separately. A 2 liter Biostat B fermentor from BBI Sartorius Inc, USA (formerly known as B.Braun Inc.) was used for this experiment. The fermentor has a working volume of 1.5 L with a automated control system and four built-in peristaltic pump-heads for dosage and nutrient addition requirements. A deionized water system was used to meet the vessel jacket specifications. Compressed air cylinders were used to maintain aerobic fermentation conditions. Cultivation was done at 37 ºC with agitation rate at 200 RPM and aeration rate set at 1 VVM (volume of air/volume of fermentation medium/minute). The pH in the medium was controlled between 7 and 7.2 by pumping NaOH or HCl. Samples withdraw from the culture fluid for analysis were separated into a cell pellet and a cell-free supernatant by 15 min centrifugation at 8000×g (Sorvall Superspeed centrifuge RC2-B, Newtown, CT, USA).

Characterization of cyanophcyin

SDS-PAGE electrophoresis

Polymers from recombinant *E.coli* were separated on an 11% sodium dodecyl sulfate-polyacrylamide gel as described by Laemmli (1970). Proteins were stained with Coomassie brilliant blue G-250.

HPLC analysis

Polymers extracted from recombinant *E.coli* cells was dissolved in 0.1 M HCl. 1 ml of sample was filtered through a 0.45 syringe filter for analysis using a HPLC system (CBM-20A system, Shimadzu, Columbia, MD, USA) equipped with a size extrusion column (Biosep SEC-S 2000), a UV detector (Shimadzu SPD-M20A) and a refractive index detector (Shimadzu RID-10A). A 0.1 M HCl solution was used as mobile phase at flow rate of 1.0 ml/min. Samples were detected at 210 nm. The HPLC column was operated at 40 ºC. Calibration was done with standard size protein purchased from Invitrogen (Carlsbad, CA, USA).

Transmission electron microscopic study

Cells were fixed in modified Karnowsky's fixative (2% paraformaldehyde, 2.5% glutaraldehyde, 1.7 mM $CaCl_2$ in 0.1 M cacodylate buffer [pH 7.4]) for 2 hours at 4 ºC. After fixation, the cells were washed twice in 0.1 M cacodylate buffer [pH 7.4] and postfixed in an osmium solution (1% osmium tetroxide in 0.1 M cacodylate buffer) overnight at 4 ºC. After this procedure, cells were washed thrice with cold double-distilled water for 5 min each, dehydrated in a series of solutions with increasing strength of ethanol (70, 70, 70, 95 and 100%). Dehydrated specimens were embedded in LX 112 medium at 42 ºC for 18 to 20 h and repeated at 60 ºC for 24 h. Embedded tissues were trimmed and sectioned. Thin sections were retrieved with copper grids and stained with uranyl acetate and lead citrate. Grids were examined and viewed in Electron Microscopy laboratory (Diagnostic Medicine Pathobiology, KSU).

Amino acid analyses

Polymers extracted from recombinant *E.coli* cells were separated in the SDS-PAGE, and then transferred to PVDF membrane with protein standard ladder. A portion of the membranes where our target protein was attached were cut and sent to Agricultural Experiment Station Chemical Laboratory, University of Missouri, Columbia. AOAC Official Method 982.30 E(a,b,c), chp. 45.3.05, 2006 was used for the amino acids analysis (Appendix-A).

Results and discussion

Characterization of cyanophycin

Polymers extracted were separated on SDS-PAGE and results obtained are present in Figure 4.1. Only the polymers obtained from recombinant BL21 (DE3) pLysS showed a major band located between 21.5 to 31 kDa. Polymers were also extracted from control BL21 (DE3) pLysS cells; however, no significant amount of cyanophycin polymer was present corresponding to molecular weight between 21.5 to 31 kDa. Protein differential results showed that the protein with molecular weight of 21.5 to 31 kDa did not match any *E.coli* protein when compared with *E.coli* protein database. For the BL21 (DE3), the polymers extracted from the recombinant or the control cells showed no major band between 21.5 to 31 kDa. It is evident that without the control of pLysS plasmid, the BL21 (DE3) harboring *cphA* cannot produce cyanophycin. The reason why recombinant BL21 (DE3) strain did not produce cyanophycin was still unclear. We speculated that the activity of cyanophycin synthetase produced by recombinant BL21 (DE3) was too low or perhaps cyanophycin synthetase produced by recombinant BL21 (DE3) was not functional. Even at sufficient cultivation time and amino acids, the culture cannot synthesize low-molecular weight cyanophycin as primers.

Mass spectrometry results (Table 4.1) showed that the average molecular weight of polymer presented in the major band on SDS-PAGE was about 29 kDa.

Cyanophycin was also characterized by HPLC, and the results are showed in Figures 4.2 and 4.3. Compared to the HPLC profile of control sample, the profile of the recombinant showed three unique peaks in the HPLC analysis. From the peak table (Tables 4.2 and 4.3); the retention time of these peaks were 7.156, 9.643, and 12.532 min, respectively. Compared with standard protein (Table 2.1, Chapter-2), molecular weight of 7.156 and 9.643 peaks was around 116.3 and 97.4 kDa. These results did not agree with SDS-PAGE and MS analyses, since the proteins corresponding to these two peaks could be some large cell debris protein. The molecular weight of the 12.532 peak was close to the 21 kDa, which agreed with the SDS-PAGE and MS results.

Amino acids analysis of cyanophycin (Table 2) showed that the two overwhelming amino acids are aspartic acid and arginine, in the ratio of 1:1 (mass basis). Besides these two major amino acids, sother amino acids are also present, such as lysine, glutamic acid, valine and so on. Lysine is the third large amino acid. According to Frey *et al*.,[11] a small part of arginine in cyanophycin could be replaced by lysine. The ratio of Asp: Arg: Lys was 1.05: 1: 0.2 (mass basis).

The possibility of thin stillage as substrate for cyanophycin production was evaluated. Polymers extracted from recombinant BL21 (DE3) pLysS and BL21 (DE3) grown in LB broth with 5 % thin stillage addition. SDS-PAGE analysis showed no major bands around 21.5 to 31 kDa (picture not shown). This result showed that thin stillage may not be a suitable feedstock for cyanophycin synthesis. The amino acids analysis of thin stillage was performed later and the

results are present in Table 4.5. Aspartic acid and the arginine content was very low in thin stillage, which explained why cyanophycin was not synthesized.

Preliminary experiments were conducted to evaluate potato waste for cyanophycin production. SDS-PAGE results (Figure 4.4) showed the polymer extracted from recombinant *E.coli* grown in 1 % casamino acids addition and 1 % potato waste as substitute for casamino acids. All of them had major bands located between 21.5 and 31 kDa, which suggested that the potato waste was an appropriate substitute for the expensive casamino acids.

Microscopy Study

Cyanophycin is also referred to as "structural granules" because of substructures visible in electron micrographs (Lang, 1968). Cells of recombinant *E.coli* grown under casamino acid addition and IPTG induction conditions were investigated by phase-contrast light microscopy (Figure 4.5). Most cells contained light refractile granules, which were cyanophycin granules. Transmission electron micrographs (Figure 4.6) of thin section of the *E.coli* cells gave a more detailed view of the cells and the granules. These micrographs revealed that some square or round light refractile compounds were inside the recombinant *E.coli* cells; however, the structure of *E.coli* control cells was much more homogenous.

Cultivation of recombinant BL21 (DE3) pLysS at 2-liter scale fermentor

Batch cultivation of recombinant *E.coli* in LB broth with glucose as the carbon source and in the presence of 1% casamino acids was performed. Figure 4.7 shows the time-course fed batch fermentation profile. Cyanophycin accumulated to 12.6% (wt/wt) of the cellular dry matter when fermentation was performed at temperature 37 ºC, aeration at 1VVM, and agitation at 200 RPM. Before IPTG induction, no cyanophycin was produced. Induction was done after 2 hours

incubation when the OD_{600} value reached 0.4. The cyanophycin content of the cells increased rapidly within the first 4 hours after IPTG induction, from 0 to 8% (wt/wt) of cell dry weight. A cell density of 2.25 g/l was obtained with a cyanophycin content of 12.6% (wt/wt) at the end of fermentation. Glucose in the medium cannot be detected after 8 hours fermentation. Compared with cell density (6.7~8.3 g/l) reported by Frey *et al* (2002), the low cell density of our culture could be due to two-phase fermentation, growth phase before IPTG induction, and production phase after IPTG induction. After IPTG induction, most energy was shunted towards protein synthesis. If we switched the induction time to later exponential phase, the cell density would have increased.

Conclusions

Recombinant *E.coli* cells were grown in LB broth with 1% casamino acids; polymer extracted from recombinant E.coli cells were characterized using SDS-PAGE electrophoresis, HPLC, TEM and amino acids analysis. Results revealed the cyanophycin synthetase produced by recombinant BL21 (DE3) was not functional, and cyanophycin cannot be synthesized in recombinant BL21 (DE3). Cyanophycin was only obtained when casamino acids was added as precursor, similar to the studies conducted by Frey *et al.,* (2002). After 20 hours cultivation, a cell density of 2.25 g /l was obtained with a cyanophycin content of 12.6 % (wt/wt). Cell density was low and is due to IPTG induction in the early exponential phase.

The potential of thin stillage and potato waste as substitute substrates for casamino acids for cyanophycin production were evaluated. Result showed that thin stillage was not an efficient feedstock for cyanophycin synthesis due to its low aspartic acid and aginine content. However, potato waste can be used to replace the expensive casamino acids in cyanophycin synthesis. The potato waste contains aspartic acid and asparagine up to 65.1 g/l, and arginine content is about

9.2 g/l (Elbahloul *et al*., 2005a). The high aspartic acid, asparagines and arginine contend are of great interest with regard of commercial production of cyanophycin.

Table 4.1 Mass spectrometry data

ID	Spectrum	Data type	Accession	Seq pI	Seq MW[kDa]	Intens. Cov[%]
7726	MS-1	MS	gi|119390347	11.5	29.2	41.2
7726	MS-1	MS	gi|223571	11.6	29.7	40.4
7726	MS-1	MS	gi|33357902	11.6	29.7	40.4
7726	MS-1	MS	gi|15803844	11.6	29.8	40.3

Table 4.2 HPLC peak table of polymer extracted from control BL21 (DE3) pLysS

Peak #	Ret. Time	Area
1	5.787	20027267
2	6.213	17306882
3	7.051	7435127
4	7.457	4726330
5	7.848	7135356
6	8.346	20694032
7	9.480	2760265
8	10.025	1573761
9	10.319	4835735
10	10.833	7777867
11	11.562	1607393
12	12.066	5600891
13	13.509	9399

Table 4.3 HPLC peak table of polymer extracted from recombinant cell BL21 (DE3) pLysS

Peak #	Ret. Time	Area
1	5.786	64664962
2	**7.156**	**61541039**
3	7.820	8867406
4	8.309	24263023
5	9.490	1209786
6	**9.643**	**1948585**
7	10.025	1616992
8	10.322	5138246
9	10.840	7595941
10	11.532	1598475
11	12.062	4075286
12	**12.532**	**1247157**
13	13.515	404271

Table 4.4 Amino acids analysis of cyanophycin ("as is" basis)

Amino acid	µg /sample	Amino acid	µg/sample
Aspartic acid	**42.79**	Leucine	3.65
Arginine	**40.79**	Histidine	0.63
Lysine	**8.24**	Proline	0.72
Glutamic acid	4.46	Taurine	0.7
Valine	2.51	Threonine	1.51
Phenylalanine	2.02	Isoleucine	2.16
Methionine	0.18	Tyrosine	0.05
Hydroxylysine	0.05	Serine	1.52

Table 4.5 Amino acid analysis of thin stillage

Amino acid	%	Amino acid	%
Isoleucine	0.01	Ornithine	0.00
Leucine	0.02	Lysine	0.03
Arginine	0.03	Histidine	0.01
Tyrosine	0.01	Tryptophan	<0.04
Phenylalanine	0.01	Hydroxylysine	0.01
Crude protein	0.84		

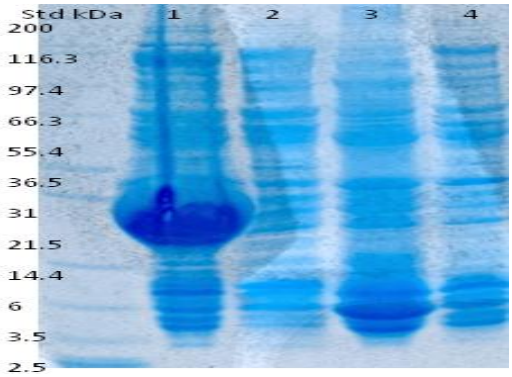


Figure 4.1 SDS-PAGE analysis for polymers from recombinant *E.coli* cells
lane1: polymer from IPTG induced recombinant BL21 (DE3) plysS; lane 2: polymer from IPTG induced BL21 (DE3) pLysS without *cphA* gene; lane 3: polymer from IPTG induced recombinant BL21 (DE3); lane 4: polymer from IPTG induced BL21 (DE3) without *cphA* gene

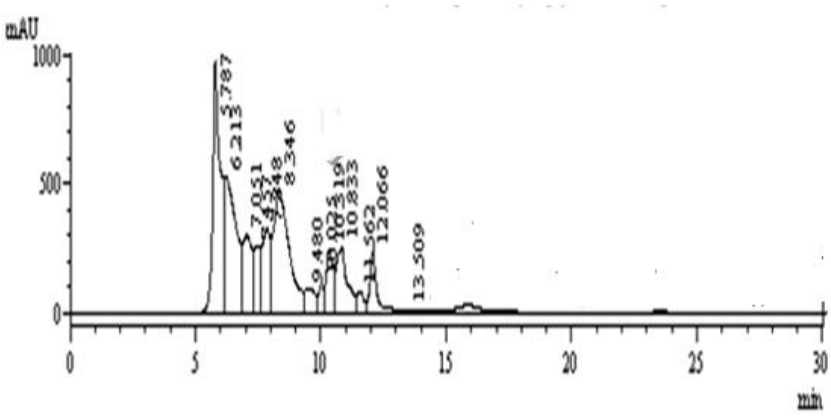


Figure 4.2 HPLC profile of polymer extracted from control cell BL21 (DE3) pLysS

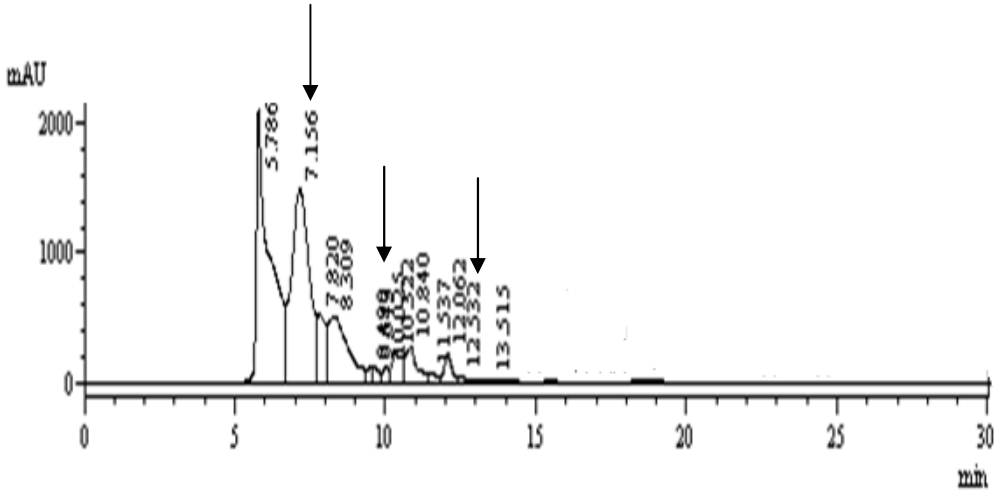


Figure 4.3 HPLC profile of polymer extracted from recombinant BL21 (DE3) pLysS

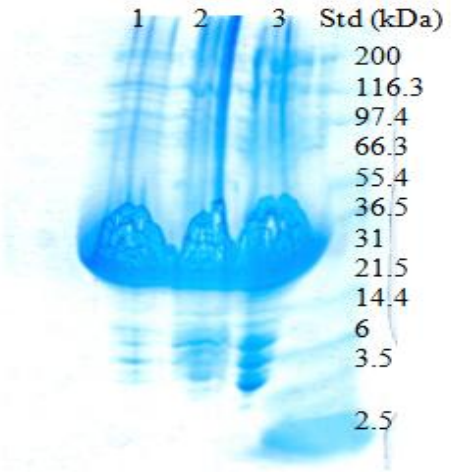


Figure 4.4 **Evaluation of potato waste as substitute for cyanophycin synthesis**
Lane 1-2: cyanophycin extracted from recombinant BL21(DE3) pLysS using 1 % casamino acid addition; lane3: cyanophycin extracted from recombinant BL21 (DE3) pLysS using 1 % potato waste as substitute

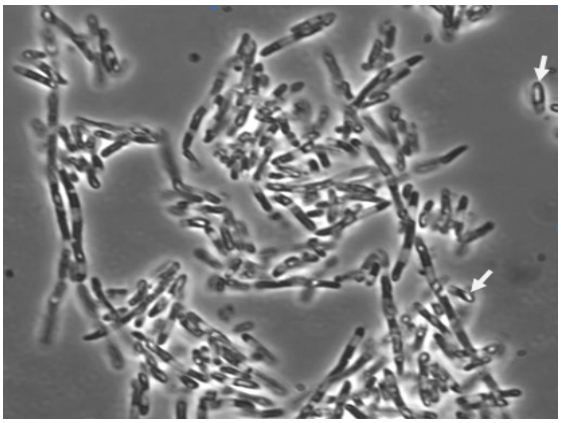
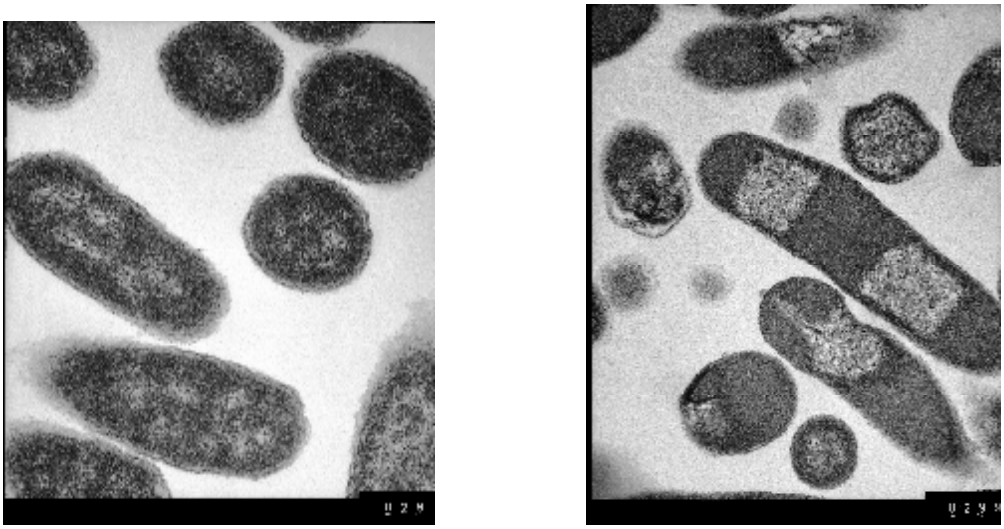


Figure 4.5 Phase contrast photomicrograph of IPTG induced recombinant BL21 (DE3) pLysS



(A) (B)

Figure 4.6 Microscope inspection of IPTG induced recombinant BL21 (DE3) pLysS
(A) control (B) recombinant

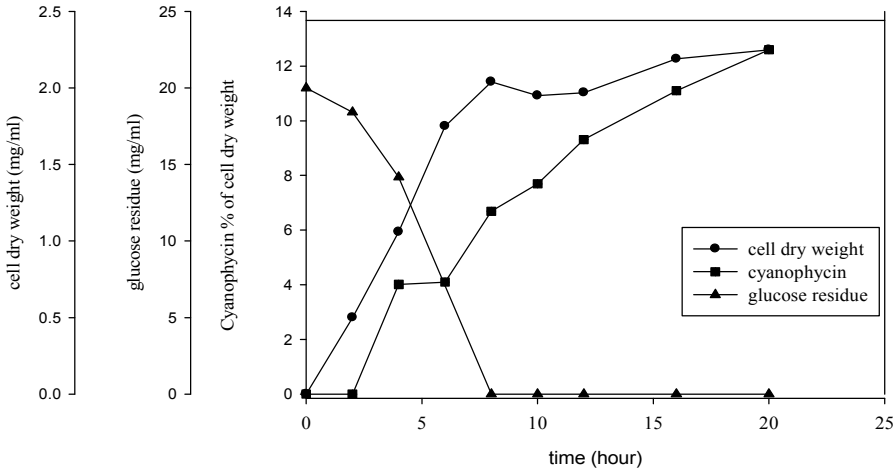


Figure 4.7 Cultivation of recombinant BL21 (DE3) pLysS at the 2-liter scale fermentor

CHAPTER 5 - Conclusions and future work

Cyanobacteria accumulates limited amount of cyanophycin. *A. variabilis* ATCC 29413 was grown in a 5-L fermentor, ribosomal inhibitor chloramphenicol was added to a final concentration of 10 μg/ml. The yield of cyanophycin was very low (0.27% of dry cell mass) without chloramphenicol addition; whereas, yield increased to 2.43% of dry cell mass after chloramphenicol was added. Chloramphenicol inhibits the growth of culture; cell density decreased from 4.18 g/l to 2.57 g/l at the end of fermentation after chloramphenicol was added. Possible explanation for this phenomenon is that some ribosomal synthesized protein is inhibited by chloramphenicol, and these proteins are necessary for the growth, therefore resulting in decreased growth rate.

Because of low production of cyanophycin by *A. variabilis* ATCC 29413, cyanophycin synthetase gene (*cphA*) was cloned in *E.coli* cells in order to develop a high expression and fast growing strain. A 2.7kb *cphA* from *A. variabilis* ATCC29413 was amplified by normal PCR, cut by SacI and PstI restriction enzymes, ligated with pET 45b+ vector and introduced to BL21 (DE3) pLysS and BL21 (DE3) *E.coli* cells. Positive colonies were selected by plasmid analysis, colony PCR, and DNA sequencing. Both *E.coli* strains produced cyanophycin synthetase; BL21 (DE3) produced more enzyme based on the western blot results. Cyanophycin synthetase (CphA) was produced by the *E.coli* gene-expression system as a C-terminal his-tagged protein. Western blot result indicated the molecular weight of cyanophycin synthetase was 98 kDa. IPTG induction time was critical for cyanophycin synthetase formation. Better enzyme production was obtained at 4 hours induction time.

Polymers produced by recombinant BL21 (DE3) pLysS and BL21 (DE3) was characterized using SDS-PAGE, HPLC, TEM and amino acids analyses. Recombinant BL21

(DE3) was not able to produce cyanophycin, which would be the low activity or even nonfunctional cyanophycin synthetase that cannot synthesize low molecular weight cyanophycin as primer. Casamino acids as precursor were necessary for cyanophycin biosynthesis. Cell density of recombinant BL21 (DE3) pLysS reached 2.25 g/l and cyanophycin content was 12.6 % (wt/wt) after 20 h fermentation.

In order to reduce the cost of cyanophycin production, thin stillage and potato waste were evaluated as a substitute for expensive casamino acids. Results showed that thin stillage made in Bioprocessing laboratory (BIVAP, Grain Science & Industry Department, KSU) was not an efficient feedstock for cyanophycin synthesis due to its low amino acids content and lack of aspartic acid. However, potato waste can be used to replace the expensive casamino acids in cyanophycin synthesis due to its high soluble polypeptides and amino acids content.

Future work will focus on the strain robustness and plasmids stability improvement; optimization of fermentation conditions; and application of cyanophycin granule. After several generations, *E.coli* cells may lose the recombinant plasmids. Robust strain should be selected and plasmids should be maintained inside cells. Fermentation conditions need to be further optimized in order to increase cyanophycin production. Fed-batch or continuous fermentation model may be applied. Optimal IPTG final concentration and induction point will be determined. Evaluation of various biomass resources for production of cyanophycin may also need to be conducted to lower the cost. The applications of cyanophycin granule itself are still unknown till date; investigation of its application will open more opportunities to this unique polymer.

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Appendix A - AOAC official method 982.30 E(a, b, c), chp.45.3.05,2006

Acid hydrolysis

Place 0.1 mg test protein in hydrolysis tube, add 10 ml 6 M HCl and mix. Freeze in dry ice- alcohol bath. Draw and hold vacuum of ≤ 50 mm for 1 min; seal tube under vacuum. Hydrolysate through Whatman No. 1 paper; rinse tube 3 times with H_2O and filter each rinse. Dry filtrate at 65 ºC under vacuum. Dissolve dry hydrolysate in volume of buffer appropriate for amino acid analyzer. Store hydrolysate for more than a week before analysis. Use this hydrolysate to determine all amino acids except methionine, cystine and/or cysteine, and tryptophan.

Performic acid oxidation followed by acid hydrolysis

Place 0.1 mg test portion in hydrolysis tube, add 2 ml cold performic acid, and let sit overnight at 0-5 ºC. Add 3 ml cold HBr and 0.04 ml 1-octanol (antiform); immediately mix contents 30 s in ice –water bath and evaporate to dryness at 40 ºC under vacuum. Add 10 ml 6 M HCl to tube and perform acid hydrolysis as described above. This treatment will quantitatively convert methionine to methionine sulphone and cystine and/or cysteine to cysteic acid. Use this hydrolysate to determine methionine (MET) and cystine/cysteine (CYS).

Alkaline hydrolysis

Place 0.1 mg test portion into glass hydrolysis tube having Nalgene polypropylene centrifuge tube as internal liner. Add 25 mg hydrolyzed potato starch (omit if product is high in starch). Add 0.6 ml fresh 4.2 M NaOH and 0.04 ml 1-octanol. Mix contents 2 min under partial vacuum. Freeze tube contents in dry ice-alcohol bath. Draw and hold vacuum ≤50 mm 1 min;

seal tube while under vacuum. Hydrolyze 22 h at 110 ± 1ºC, cool, open tube, and transfer contents to 5 ml volumetric flask containing sufficient cold 6 M HCl to neutralize hydrolysate; dilute to volume using buffer appropriate for amino acid analyzer. Centrifuge or filter hydrolysate and store frozen. Use this hydrolysate to determine tryptophone (TRP).