EXPRESSION OF RECOMBINANT *THERMUS AQUATICUS* DNA POLYMERASE GENE

By

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CERTIFICATE

This is to certify that the dissertation entitled

"EXPRESSION OF RECOMBINANT *THERMUS AQUATICUS* DNA POLYMERASE GENE "

is the bona fide record of research work done by

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LIST OF ABBREVIATIONS

DNA	Deoxyribonucleic acid
Taq	*Thermus aquaticus*
SDS- PAGE	Sodium Dodecyl Sulphanate Polyacrylamide Gel
PAGE	Polyacrylamide Gel
LB	Lauria Bertani
IPTG	Iso- propyl-1-thio-β-D-galactopyranoside
PCR	Polymerase Chain Reaction
Taq Pol I	Thermus Aquaticus DNA Polymerase
Taq I	Thermus Aquaticus endonuclease
dNTP	Deoxyribonucleoside Triphosphate
bp	Base pair
kb	Kilo base pair
KF	Klenow Fragment
NaCl	Natrium Chloride
APS	Ammonium Persulphate
TBE	Tris Base- EDTA
rpm	Rotor per minute

1.0 ABSTRACT

Taq DNA polymerase derived from the extreme thermophillic microorganism, *Thermus aquaticus* is very useful in Polymerase Chain Reaction (PCR) in which high temperature stable Deoxyribonucleotide acid (DNA polymerase is needed in amplifying a specific DNA fragment. It is also useful in DNA sequencing. For this purpose, many techniques of cloning and expression of this enzyme were practiced to obtain a high performance enzyme. The purification methods were designed to retrieve a high enzyme yield. In this method, we have tried to express the recombinant Taq Pol I enzyme in different expression systems to select the best host for the protein expression. The host BL-21 was selected because of the additional property within it, a pLysS plasmid for tighter protein expression but simpler purification method as adapted from Grimm et. al (1995). Protein expression was induced over a 12 hour period using IPTG before it was viewed in a large SDS- PAGE. There were bands along a marker indicating a complete protein expression as the weight of Taq DNA polymerase is 94 kD.

2.0 ABSTRAK

DNA polymerase yang didapati di dalam mikrooorganisma themofilik, *Thermus aquaticus* amat berguna dalam teknik 'Polymerase Chain Reaction (PCR)' memandangkan enzim yang digunakan dalam mengamplifikasikan fragmen DNA perlu stabil pada suhu tinggi. Selain itu, enzim Taq DNA polymerase ini amat berguna dalam 'DNA sequencing'. Dalam penghasilan enzim yang berkualiti tinggi, banyak aktiviti pengklonan dan pengekspresan dijalankan dalam pelbagai sel hos. Dalam kaedah ini, kami telah mengkaji pengekspresan protein rekombinan Taq DNA polymerase dalam pelbagai system pengekspresan untuk memilih sistem pengekspresan yang terbaik. Sel BL- 21 telah dipilih kerana plasmid pLysS yang terkandung di dalamnya boleh memberi ekspresi protein yang ketat tetapi teknik purifikasi yang lebih mudah seperti yang diadatasi dari Grimm et. al (1995). Protein diinduksi selama 12 jam menggunakan IPTG seelum melihat keputusan pada gel SDS- PAGE. Didapati terdapat jalur pada kedudukan 94 kD yang menunjukkan penghasilan protein Taq DNA polymerase yang lengkap.

3.0 INTRODUCTION

The bacterium *Thermus aquaticus* (*Taq*) was first discovered in several hot springs in the Great Fountain area of the Lower Geyser Basin, Yellowstone National Park, Wyoming, USA (Figure 1.1). Due to its hyperthermophillic nature, this bacterium could survive in very high temperate ranging between 50°C to 80°C. The temperature range is much higher than the normal temperate needed for survival of other bacteria as the highest temperature limit discovered till then was 73°C, which was for photosynthetic bacteria such as cyanobacteria. Yet the most optimum temperature for the activity of *Thermus aquaticus* is at 70°C. As its temperature range somewhat overlaps that of the photosynthetic bacteria found abundant in Yellowstone springs, it lives in association with cyanobacteria, obtaining its energy for growth from the photosynthesis of these organisms.

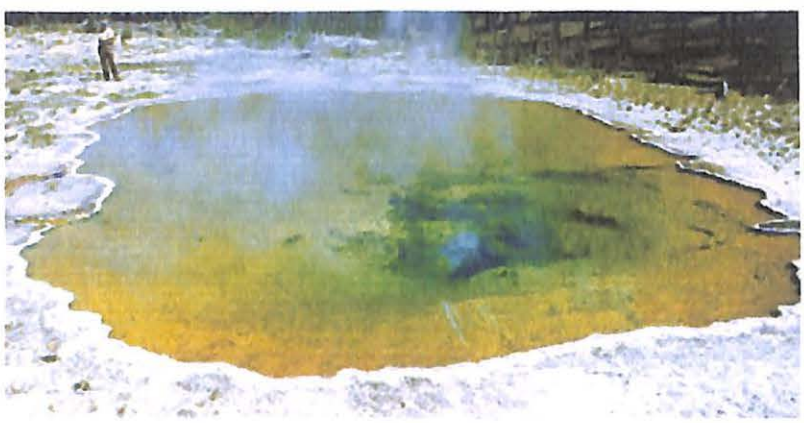

Figure 1.1

The large spring of the Lower Geyser Basin

One of the first papers published on *Thermus aquaticus* (*Taq*) DNA polymerase was on the finding of the protein itself by Chien et. al (1976). In this paper a stable DNA polymerase has been purified from *Thermus aquaticus* strain YT- 1 and studied. The methodology involves growing the bacteria strain in a defined mineral salts medium at pH 8.0 and at the temperature of 75°C. The enzyme is then extracted by rupturing the cell wall using an ultrasonic oscillator and spinning the extracts. The supernatant fluid served as the crude extract and the protein concentration is determined through the method of Lowry et. al (1951). Enzymatic assays were done to determine the exonuclease, alkaline phophomonoesterase and alkaline phosphodiesterase I activity but was found that the DNA polymerase was free from all of these. Purification is done through column chromatography method but 95% of enzymatic activity was lost during this procedure. To get an estimate of the purity prepared, polyacrylamide gel elctrophoresis (PAGE) is done and the enzyme obtained is not homogenous but contains a fraction of BSA from the purification method. The molecular weight estimation through sucrose gradient method indicates the protein was approximately 68,000 kD which is similar to the results obtained through the gel filtration method which was 63,000 kD. The enzyme was found to require all four deoxyribonucleaside triphophates (dNTPs) as well as DNA and Mg^{2+} and its optimum temperature is found to be at 80°C.

Studies of extracting enzymes from *Thermus aquaticus* did not end there as Sato et. al. (1977) at the very next year successfully extracted and purified the a sequence specific endonuclease, Taq I from the thermophilic

microorganism. For the purification of the protein, *Thermus aquaticus* cell culture are thawed in buffer A containing Tris / HCl as well as mercaptoethanol and then disrupted by means of French pressure cell. The cell extract is then obtained through centrifugation and suspended in phosphocellulase. Column chromatography method is followed next by using an Amicon cell with a UM-10 membrane. The enzyme yield though is not very high in comparison with endonucleases enzymes that are extracted from other bacterial strains. This could be due to the non- binding property some of the cell extracts have towards phosphocellulose.

Studies on *Taq* DNA polymerase structure was also done to understand the function and properties of this enzyme. From the crystal structure (Figure 1.2), it was found that the space between the "fingers" and the "thumb" is just the right size for a DNA helix. But surprisingly, DNA actually fits into the palm when the enzyme is at work. The enzyme contains three separate active sites. The polymerase site, synthesizes the new strand by adding nucleotides. The 3'-5' exonuclease site, proofreads the new additions. The polymerase from *Thermus aquaticus* does not have this proofreading ability--perhaps the heat in which it lives performs the same function. At the bottom is the 5' exonuclease site that later removes the small RNA fragments that are used to prime DNA replication.

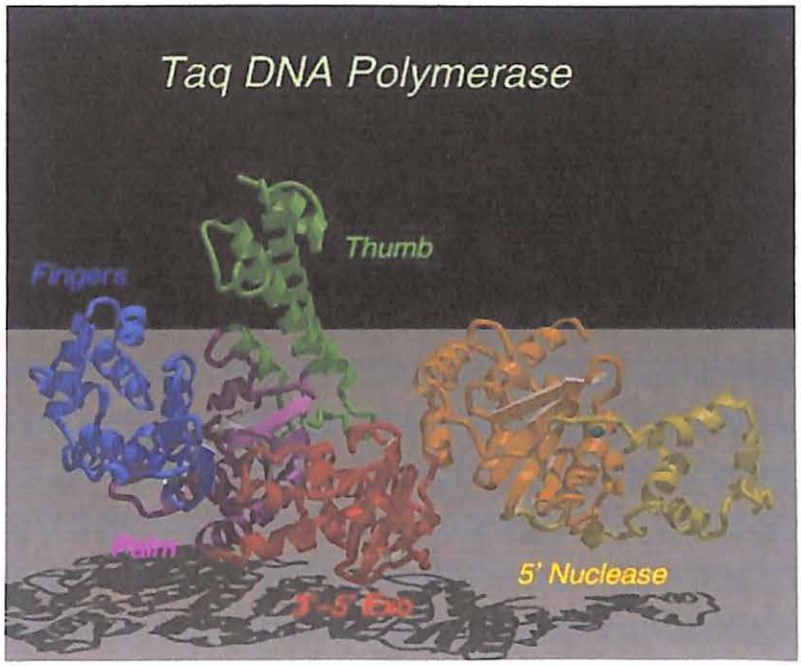

Figure 1.2

Crystal structure of Taq DNA polymerase

From two publications by Steitz et. al. in the year 1995 and 1996, the structure of DNA polymerase from *Thermus aquaticus* (Taq polymerase) was found to be homologous to *Escherichia coli* DNA polymerase I (Pol I) and likewise has domains responsible for DNA polymerase and 5' nuclease activities. The structures to the polymerase domains of *Taq* polymerase and of the Klenow fragment (KF) of Pol I are almost identical, whereas the structure of a vestigial editing 3'-5' exonuclease domain of *Taq* polymerase that lies between the other two domains is dramatically altered, resulting in the absence of this activity in the thermostable enzyme. The structures have been solved for editing complexes between KF and single-stranded DNA and for duplex DNA with a 3' overhanging single strand, but not for a complex containing duplex DNA at the polymerase active-site. Here the co-crystal structure of *Taq* polymerase with a blunt-ended duplex DNA bound to the polymerase active-site cleft; the DNA neither bends nor goes through the large polymerase cleft, and

the structural form of the bound DNA is between the B and A forms. A wide minor groove allows access to protein side chains that hydrogen-bond to the N^3 of purines and the O^2 of pyrimidines at the blunt-end terminus. Part of the DNA bound to the polymerase site shares a common binding site with DNA bound to the exonuclease site, but they are translated relative to each other by several angstroms along their helix axes.

Among the undistinguished functions of Taq Pol I is as an amplifier in Polymerase Chain Reaction (PCR) and in DNA sequencing. In PCR though, it was found that discrepancies occur due to activation of the enzyme at lower temperatures. To avoid this problem, hot start PCR was created to provide with activation of *Taq* polymerase only when high temperature is reached (70°C) and ctivity is inhibited at room temperatures. The latest advancements in hot- start PCR brought to many modifications of the *Taq* DNA polymerase enzyme in order to create an enzyme which has 0% activity at low temperatures of 20°C but regain its maximum activity at high temperatures of 70°C. For this purpose, monoclonal antibodies against the thermophilic enzyme such as AntiTaq by Novagen are used to inhibit the activity of the enzyme till the temperature reaches 70°C. Yet, it was found that this method could only partially suppress the activity of the enzyme (80% - 95%). Thus, Milko et. al. (2003) began their search in the mutagenized library to identify cold sensitive *Taq* DNA polymerases that have suppressed enzymatic activity at room temperature but stable enzymatic activity at high temperature of the PCR cycle. The Klentaq library was studied for this purpose, which had enzymes with NOterminal deletions of the Taq DNA polymerase. Firstly, all 3,800 mutant clones were

screened for cold sensitivity phenotype by an in colony DNA polymerase assay. Promising candidates were then selected and subjected to additional screening for lower incorporation at 37 and 42°C but good incorporation at 68°C. Sequential analysis was then done to in which 3 adjacent amino acids were found to be related to cold- sensitivity. Dual temperature assay and thermostability tests were carried out before testing the mutants in hot start PCR. The fidelity of these mutants were found to be better than the parent Klentaq giving rise to possibilities of more probable mutants that are also cold-sensitive yet optimal during hot- start PCR.

Expression and purification of recombinant Taq DNA polymerase started almost immediately after the discovery of the protein. At year 1989, Lawyer et. al. attempted to clone and express the gene in *Escherichia coli*. The recombinant gene was recovered from a λgty11; Taq library using antibody probing. The gene is then restricted with *Eco*RI forming four different sizes of fragments. The 115 bp fragment was to be sub cloned into Genescribe Z vector pTZ19R to be used for screening in the λCh35; Taq. From here 9 candidates were isolated and one is selected based on its correspondence with Hind III fragment that was hybridized with *Taq* genomic in Southern Blot. 4 of its detectable fragments are further subcloned into pFC82.35 and pFC82.2 before proceeding with protein induction and enzymatic activity assay. A full length Taq Pol I gene was assembled in pLSG1 in DG101 and induced for protein expression but the desired result which is a detectable induced band is not seen.

In 1993 Lawyer et. al. cloned Taq Pol I containing 832 amino acids and a truncated form containing 544 amino acids (Stoffel fragment) into a plasmid expression vector which utilizes the strong bacteriophage lambda P promoter. Upon heat induction, proteins were produced with 3% of total arising from Stoffel fragment (9) and 1-2% arising from Taq Pol I. Protein purification involves cell lysis, phenyl sepharose column chromatography and heparin sepharose column chromatography. The yield obtained on Taq Pol I enzyme 3.26×10^7 units 165 g of wet weight cell paste. The enzyme has maximal activity at 75°C to 80°C with $MgCl_2$ as cofactor and polymerization-dependent 5' to 3' exonuclease activity.

Most of the *Taq* DNA polymerase enzymes avalaible commercially is expressed in pTaq expression vector. The method was discovered from Engelke et. al. (1990) and was significant due to the overproduction of the enzyme. The *Taq* DNA polymerase gene was amplified and unique *Eco*RI and *Bgl*II restriction endonuclease sites were created at two ends of the fragment. After further amplification of this modified gene, the product was cleaved with *Eco*RI and *Bam*HI and isolated at low melting points agarose gels. The fragments are then ligated into expression vector pTTQ 18 (Amersham) that have been restricted with this enzyme. The recombinant plasmid is then transformed into E. coli strain DH1 and induced for protein expression with Iso-propyl-1-thio-β-D-galactopyranoside (IPTG). Protein overproduction was monitored with denaturing polyacrylamide gel. Purification of protein was done through column centrifugation method using BioRex70 ion exchanger column.

From the method described from Engelke et. al.(1990), two journals were further published on modified purification methods. One of such is by Pluto et. al. (1993) in which mentioned about the modification on the bacterial strain used, induction and protein concentration during isolation has been optimized. In this method, *E.coli* strain INV1alphaF' (Invitrogen) was used as an expression host. Protein expression under the control of *tac* promoter is done through IPTG induction for eleven hours cells are then lysed, precipitated and the protein is recovered through action of $(NH_4)_2SO_4$ in which protein precipitated. The activity of the purified enzyme was found to be 1 unit/ 100ng of protein.

Another paper on modified protein purification is by Grimm et. al. (1995). In this paper, the property of pLysS plasmid is exploited to cater a much simpler method of protein purification. Here, competent cells, BL- 21 with pLysS plasmid become the expression host for pTaq vector to express the Taq Pol I protein. Once expressed, purification method is done by resuspending in Buffer A (50mM of Tris- HCl, 50mM glucose, 1mM of EDTA). The suspension is then subjected to two cycles of freeze and thaw method (at -70°C and 70°C). The cell debris was then removed from the lysate by centrifugation and the supernatant containing enzyme is dialysed against excess of buffer before storage at -20°C.

We have started this research in bid to produce our own *Taq* DNA polymerase to be used in PCR methods. The enzyme we are working for was to be of high enzymatic activity with no decrease in activity for months. Also what we were aiming for was for a simple purification step in obtaining a clean yield therefore, it was decided we employ the similar method from Grimm et. al (1995). We received pTaq construct {as described by Engelke et.al (1990)} as a

gift and continued our methodology from there by first increasing the copy number of the construct following replication in TOP 10. Expression is then studied using different systems and to be continued with isolation and purification. Finally, enzymatic activity of the protein was to be analyzed through PCR titrations and comparison with other commercial *Taq* enzymes.

4.0 MATERIAL

4.1 pTaq. A plasmid as described in Engelke et. al (1990). containing Taq Pol I gene. Originally was an expression vector pTTQ18 (Amersham) which was ligated with the gene of interest (Taq Pol I) at restriction sites *Bam*HI and *Eco*RI. Plasmid map could be referred in Appendix 10.3.

4.2 Stab culture. Believed to be *Taq* clone that is cultured in solid media. Also received together with pTaq and believed to be as described in Engelke et. al. (1990).

4.3 Primers. Both forward and reverse primers were initially purchased from Research Biolabs for amplification of the Taq Pol I gene. The sequences of the primers were determined from the *Taq* sequence (refer to Appendix 10.2). The forward primer has the following sequence:

5' ATGAGGGGGATGCTGCCCCTC 3'

The reverse primer has the following sequence:

5' TCACTCCTTGGCGGAGAGCCA 3'

4.4 Preparation of Lauria Bertani (LB) plain Agar. Used for culture of competent cells. Firstly 7.5g of peptone (MERCK), 2.5g of yeast extract (OXOID) and 5g of Natrium Chloride (NaCl {AMRESCO}) is added into a clean beaker. 250mL of dH_2O is added to mixture and mixed well using a magnetic

stirrer. Once all are dissolved, pH is checked and made up to 7.0 (Add alkali if pH is too low). 7.5g of agar (AMRESCO) is measured and placed in 500mL Duran Bottle. Mixture is added into bottle and solution is mixed well. The media is then autoclaved at 121°C for 15 minutes. Autoclaved media are then left to cool till temperature reaches 40°C meanwhile plates are labelled. Using sterile technique, the media is poured till reaches half of the depth of the plate. The agar is left to solidify and cool overnight at room temperature. The next day, agar is checked for contamination and placed in cold room if sterile.

4.5 Preparation Lauria Bertani (LB) Agar with added Chloramphenicol. Same method as is employed except addition of 500µL of chloramphenicol (34mg/ mL) giving a concentration of 34 µg/ mL just before pouring plate.

4.6 Preparation of Lauria Bertani (LB) Agar with added Chloramphenicol and Ampicillin. Same method is employed except addition of 500µL of Chloramphenicol (34mg/ mL) and 500 µL of Ampicillin (100mg/ mL) giving a concentration of 34 µg/ mL of Chloramphenicol and 100µg/ mL of Ampicillin just before pouring plate.

4.7 Preparing Resolving Buffer for Sodium Dodecyl Sulphate Poyacrylamide Gel (SDS- PAGE). 36.6g of Tris Base and 0.8g of SDS is added into 150mL of ddH_2O. All mixed well using magnetic stirrer. pH is made up to 8.8. Volume is then made up to 200mL.

4.8 Preparing Stacking Buffer for SDS-PAGE. 12.0g of Tris Base and 0.8g of SDS is added into 150mL ddH_2O. All mixed well using magnetic stirrer. pH is made up to 6.8. Volume is then made up to 200mL.

4.9 Preparation of 10% Ammonium Persulphate (APS). Quantity needed is calculated. For every 100mL prepared, 10g of APS is dissolved into ddH_2O. Therefore weight needed is calculated using $M_1V_1 = M_2V_2$ formula.

4.10 Preparation of Running Buffer. 3g of Tris base, 14.4g of Glysine and 1g of SDS is added into 500mL of ddH_2O. All mixed well using magnetic stirrer. pH is made up to 8.3. Volume of buffer is then added to 1L.

4.11 Preparation of Sample Buffer. 0.76g of Tris Base, 10mL of Glycerol and1g of SDS is added into 20mL of ddH_2O. All mixed well using magnetic stirrer. pH is made up to 6.8. Volume is then added to 50mL. Buffer is filtered and stored at 4°C.

4.12 Preparation of Commassie stain. 1g of Commassie Blue is added into 325mL of ddH_2O. 125ml of isopropanol is then added.

4.13 Preparaion of Destainer. 100mL of Acetic acid and 100mL of Isopropanol is added into 800mL of dH_2O.

4.14 Nucleospin Plasmid extraction Kit. For plasmid extraction from cells.

5.0. METHODOLOGY

This research was started using primarily two materials that were originally described by Engelke *et. al* (1990) which were a stab culture containing the *Taq* clone and plasmid containing called pTaq. We have then carried on procedures to ensure the presence of this gene and then later proceeded with the protein expression and purification.

5.1 Reviving the Taq clone. Using the stab culture given, we have tried to revive the clone by plating it on agar media and preinoculating in broth media using aseptic technique. The type of media used here is Lauria Bertani (LB) containing Ampicillin (100µg/ mL) for *Taq* clone selection and LB plain as control. Plates and broths were kept overnight in incubator at 37°C for growth. Observation is then made the next day.

5.2 Preparing a colony lysate for Polymerase Chain Reaction (PCR). A colony believed containing the *Taq* clone is taken and added in 100 µL of distilled water (dH20). Then lysate is prepared by boiling the suspended colony for 10 minutes and a brief spin.

5.3 Preparation of primers. Primers are reconstituted before usage as they were delivered in lyophilised form. Before proceeding the primers are spin down at 12, 000 rotor per minute (rpm) for 3minutes. First stock solution is prepared

by adding 100µL of dH_20 into dry primers. Then working solution of concentration 1pmol/ 10 µL was prepared to be used readily for PCR.

5.4 Preparing recombinant plasmid diluent 1/ 50. As the concentration of plasmid received is unknown, a dilluted product was prepared by adding 1µL of the received plasmid onto 49 µL of dH20.

5.5 Running PCR. Master mix was prepared for five reactions. 4 samples are used with each tube containing 18 µL of Master mix excluding DNA template.

PRODUCT	AMOUNT
dH_20	67.65 µL
10 x PCR buffer + MgCl2	10 µL
dNTP (10 mM)	1.6µL
Forward primer (20 pmol)	5 µL
Reverse primer (20 pmol)	5 µL
Taq DNA polymerase (5U / µL)	0.75 µL
TOTAL	90 µL

Table 5.1

PCR products

After aliquoting mix into into tubes, sample is added.

Tube Number	Sample
Tube 1	2 µL of Lysate
Tube 2	2 µL of recombinant plasmid (nett)
Tube 3	2 µL of recombinant plasmid (1/ 50)
Tube 4	None added (negative control)

Table 5.2

PCR Samples

After a brief spin and addition of mineral oil, PCR program was set as follows:

Temperature	Time	Cycle
95° C	3 minutes	Once
95° C	1 minute	Cycle repeated 30 times
50° C	3 minutes	
72° C	1 minute	
72° C	5 minutes	Once
72° C	Hold	Hold

Table 5.3

PCR Program

5.6 Running agarose gel electrophoresis. 1.0 % agarose gel (50mL) was prepared using agarose powder and 0.5% Tris Base- EDTA (TBE) buffer. An 8 lane comb is used for this small sized gel and Ethidium bromide was added before leaving the gel to solidify. Once solid, the comb is removed carefully and the gel is placed in the electrophoresis tank filled with 0.5% TBE buffer as running buffer. Samples were mixed with loading buffer before pipetted into gel lanes. Ladder markers of 1 kb (kilo base pair) and 100 bp (base pair) plus were added to view size of separated sample bands. The gel was then run at 150V for the first five minutes then reduced to 100V for the next hour. Once done, the gel was removed and placed in the ChemImager to view result by exposing the gel to Ultra Violet light for a few seconds. Image was captured and saved. Note: Gloves are to be used at all times while preparing and handling gel because it contains Ethidium Bromide which is carcinogenic.

5.7 Preparation of competent cells. Three types of competent cells are used in this research which were TOP10, BL-21 and DH5α. Preinoculum of the colonies into 2mL LB broth were done the night before to prepare a bacterial culture. LB broths with specific antibiotics (Ampicilin 100μg/ mL and Chloramphenicol 34μg/mL) were added to ensure these host cells could not

grow in the presence of these antibiotics (negative control). The next day, 50µL of the broth with growth were inoculated into 10mL LB broth and left in shaker at 37°C between 3 to 4 hours to grow. Optical density (OD) was checked after the time period. Once OD reached 0.6, the growth of the cells were halted by placing it in ice. Then these suspended cells were spun down at 3,000 rpm for 10 minutes at 4°C in 15mL centrifuge tubes. The supernatant was discarded and the pellet was dislodged from the bottom of the tube by gentle tapping. 1.2mL of Magnesium Chloride 100mM is then added and the pellet is resuspended by gentle tapping. The tube was kept in ice for 45 minutes before centrifuging again at 3000 rpm for 10 minutes at 4°C. Once again the supernatant was removed and the pellet was dislodged by gentle tapping. 0.6mL of Calcium Chloride was then added and the pellet was resuspended by gentle tapping. The tube was kept in ice for 45 minutes (Note: Magnesium Chloride and Calcium Chloride must always be kept in ice). Sterilised glycerol stock was added into the cells suspension in cases of future uses. The amount added was 15% of total Calcium Chloride used which in this case was 450µL. 150µL was then aliquoted into Eppendorf tubes and stored at -70°C (Up to three months).

5.8 Plasmid Extraction. The kit used for plasmid extraction is the Nucleospin Plasmid Extraction Kit. In this method, the colonies containing the needed plasmid were preinoculated the day before in 10mL LB broth and incubated in shaker at 37°C overnight. The next day, the cells were collected by spinning the culture down in 2 mL Eppendorf tubes at 12, 000 rpm at 4°C. Once all cells were collected, the pelleted cells were then resuspended in sterile

saline before continuing with plasmid extraction protocol. In this protocol, cells were lysated in alkaline/ SDS buffer and the genomic DNA were precipitated and supernatant cleared by simple centrifugation step. Lysate was then trasfererred into spin column and loading buffer was added. This buffer containing chaotropic salt breaks the hydrate shell of the genomic DNA and the DNA binds freely to the silica membrane at the bottom of the spin column. A wash step followed by reaction with elution buffer provides with a purified plasmid as the end result. Before continuing with the next step, the plasmid was quatitified using spectrometer at series of 1/ 50 µL dilution of dH_20.

5.9 Restriction analysis. This method is done to digest the circular plasmid at specific restriction sites to produce linear DNA strands. The enzyme used here is *Bgl*II, *Bam*HI and *Eco*RI from Fermentes. The digestions here were both single digestion (only one type of enzyme used) and double digestion (two types of enzymes used). Type of buffer used was determined from viewing the type of buffer that provides the highest restriction activity for the enzymes used for the particular reaction. Restriction was done for 3 hours in waterbath at 37°C. The results are then later viewed by running 0.8 % TBE agarose gel electrophoresis.

PRODUCT	AMOUNT
dH_20	12µL
Buffer	4µL
Restriction enzyme Double digestion – each enzyme Single digestion	 1µL 2µL
Plasmid	2µL
TOTAL	20µL

Table 5.4

Restriction analysis

5.10 Transformation of pLysS plasmid into BL- 21. This step is essential to prepare a BL-21 host with pLysS plasmid within it. Firstly 2µL of the plasmid was added into the prepared competent cells (150µL) and kept in ice for 20 minutes. This was labelled as Test. A negative control was prepared as well which just contains the competent cells without the inserted plasmid. Then heat shock was given at 42°C for 40 seconds. Then tubes were kept in ice for another 2 minutes before adding 350µL of LB broth. The prepared product was incubated in shaker at 37°C for 20 minutes. The final product was then plated onto LB with Chloramphenicol (34µg/ mL). Growth was examined the next day and the transformation is only considered valid if there was no growth in the control plate.

5.11 Preparation of BL- 21 with pLysS competent cells. The colonies of previously transformed pLysS into BL- 21 were preinoculated the day before preparation into 2mL LB broths. The antibiotic used here for selection of these cells is Chloramphenicol (34µg/ mL). The negative control contains both Ampicillin (100µg/ mL) and Chloramphenicol (34µg/ mL). Before continuing with preparation of competent cells the next day, the negative control is checked for absence of colony growth. The preparation of competent cells is the same as mentioned before with TOP 10, DH5α and BL- 21.

5.12 Transformation of pTaq into BL- 21 with pLysS, DH5α and TOP 10. The transformation method mentioned here is alike the one mentioned above. The plasmid used here was the one described by Engelke et. al. (1990). Also

transformation was done into TOP 10, DH5α and BL- 21 without the pLysS plasmid. The antibiotic used here for pTaq plasmid selection is Ampicillin (100µg/ mL) for all transformed cells prepared with exception of pTaq transformed into BL-21 with pLysS in which both Ampicillin and Chloramphenicol is used for selection.

5.13 *Taq* DNA polymerase protein production. This is a very important step in this project as proteins are produced through induction of IPTG. Preinoculum of colonies containing the pTaq plasmid within the host cells was done the day before protein induction and incubated overnight at 37°C. All the culture were then transferred the next day into Eppendorf tubes and cells were collected by centrifugation at 12,000 rpm for 3 minutes at 4°C. After all supernatant were removed, these cells were resuspended in 100µL LB with Ampicillin (100µg/ mL). Then 40µL of resuspension in pipetted into 10mL LB broth with Ampicillin (Ampicillin and Chloramphenicol for protein induction of pTaq within BL- 21 with pLysS) and placed in shaker at 37°C for 3 hours. Two samples were prepared and labelled as 'Induced' and 'Uninduced'. After three hours of incubation, the turbidity was checked. If the O.D. reads at least 0.5, steps for protein induction is preceded. Before that, 1mL of sample was removed into an Eppendorf tube, spun down for 2 minutes at 12,000 rpm and supernatant removed. The pelleted product is believed to contain the protein expressed during incubation. The remaining 9mL culture of the 'Induced' sample was induced with IPTG and kept in shaker for the next few hours. The 'Uninduced' sample was not added anything and just placed in shaker together with the 'Induced' sample. For the next 2^{nd}, 4^{th}, 6^{th}, 9^{th} and 12^{th} hour, 1mL of the culture was removed, spun and

pelleted down to obtain the protein production till that hour. Lastly, the rest of the bulk culture was transferred into a 10mL centrifuge tube and centrifuged at 3,000 rpm for 10 minutes. All the protein collected was kept in the freezer. If needed, proteinase inactivator was added to prevent protein degradation.

5.14 Sodium Dodecyl Sulphate Polyacylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE stands for Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis. It is a technique used in biochemistry and molecular biology to separate proteins according to their size (length of polypeptide chain).The SDS PAGE gel is made up of two different gels which are resolving gel at the below and stacking gel above. There are different types of SDS-PAGE which differ in sizes. The SDS-PAGE commonly used here is the big gel since its better in separating larger proteins. Before preparing the gel, all instruments used must be wiped clean and dry including the glass plates and the instrument is assembled. Firstly the resolving gel was prepared from the following:

PRODUCT	VOLUME
30% Acrylamide + 0.8% Bis (Bio Rad)	11.75 mL
Resolving Buffer (pH 9.3)	8.25 mL
DH_2O	15mL
10% Ammonium Persulphate	150 µL
TEMED	30 µL
TOTAL	35.18 mL

Table 5.4

Contents of resolving gel

The gel media was transferred immediately in between the glass plates leaving about 6 cm for stacking gel. Once cast, the gel is left to solidify. A layer of dH_20 is added to ensure an even surface gel. Meanwhile, the stacking gel is prepared from the following:

PRODUCT	VOLUME
30% Acrylamide + 0.8% Bis (Bio Rad)	1.2 mL
Stacking Buffer (pH 9.3)	2.5 mL
DH_2O	6.1mL
10% Ammonium Persulphate	50 µL
TEMED	10 µL
TOTAL	9.86 mL

Table 5.5

Content of Stacking Gel

Gel was transferred into assemble and reside above resolving gel. Loading sample was prepared by first resuspending protein in 10mM Tris Base (pH7.4). Loading buffer was prepared from 9:1 ratio of sample buffer (0.125M Tris HCl, 4% SDS, 20% Glycerol) and mercaptoethanol. A bit of bromophenol blue is added to the solution above. A 1:1 ratio of protein sample and loading buffer was vortexed together before boiling the loading sample for 5 minutes. The loading sample was centrifuged at 12,000 rpm for 3 minutes to extract the supernatant for loading. Low molecular weight marker was used for in this gel to observe the presence of a 94 kD protein.

Before loading sample, the tank was filled with running buffer (14.4 g glysine, 3 g of Tris Base and 1g of SDS for every 1 liter of dH_20- pH 8.3). The samples were loaded using Hamilton syringe. Gel was run for 5 and half hours at 25 mAmp using Power Pac system.

After gel was run, the gel was removed carefully from adjoining glass plates and left for staining on a belly- shaker in Commassie stain solution containing 90% Commassie stain (1g of Commassie Blue, 125 mL of isopropanol and 325 mL of dH_20) and 10% of Acetic Acid. After 2 hours of

staining, the gel was destained with destainer with replacement every hour till gel was clear and bands could be clearly seen. Lastly, the gel is viewed under white light using a ChemImager.

5.15 The principle of SDS-PAGE. The main principle of SDS-PAGE is to separate protein according to its shape and size.

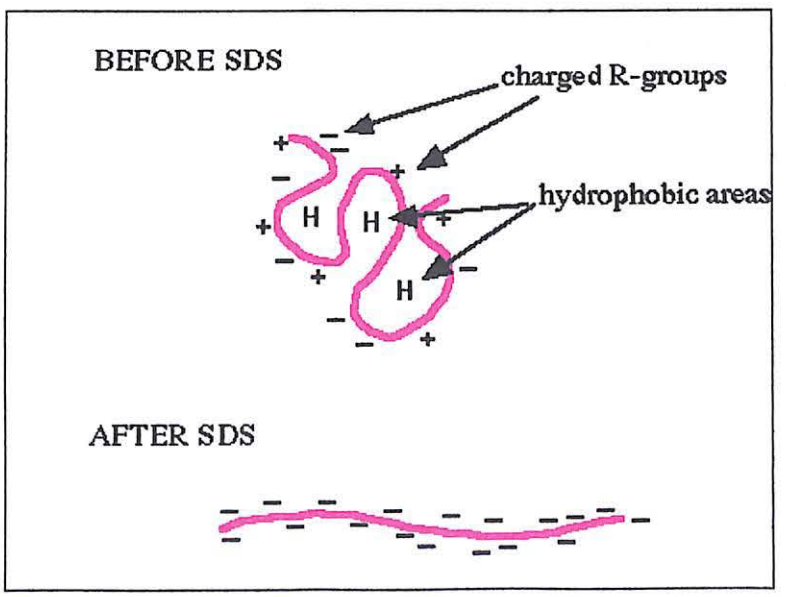

Figure 5.1

Protein Denaturation

Protein are first denatured releasing it any secondary, tertiary or quartenary structure and coated with negative charge.This is done by treating the sample to SDS (Sample Buffer)