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Cloning and Expression of a bacteriophage DNA ligase

By

Alia' Mousa AL-Manasra

In Partial Fulfillment of the Requirements for the Degree

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The undersigned hereby certify that they have read and recommend to the Faculty of Scientific Research and Higher Studies at the Palestine Polytechnic University and the Faculty of Science at Bethlehem University for acceptance a thesis entitled:

Cloning and Expression of a bacteriophage DNA Ligase

By
Alia' AL-Manasra

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in biotechnology

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ABSTRACT

Cloning and expression of a bacteriophage DNA ligase

By Alia' AL-Manasra

During the last 50 years, major advances in molecular biology and biotechnology have been attributed to the discovery of enzymes that allow molecular cloning of important genes. One of these enzymes that has been widely acknowledged for its role in the development of biotechnology is the T4 DNA ligase. This enzyme joins the DNA break in the backbone structure by creating a phosphodiester bond between 5' PO4 and 3' OH ends, in an ATP dependent multi-step reaction, thus allowing the ligation of related and foreign DNA sequences.

Due to its role in modern DNA recombinant technology, there is a high demand on DNA ligase to allow the ligation of target DNA inserts into a chosen vector as part of DNA cloning technology.

This project aimed to isolate a bacteriophage from which the DNA ligase gene would be cloned and expressed. Bacteriophage was isolated from samples collected from Hebron municipal wastewater and the gene was successfully cloned and expressed in *DH5α E. coli* strain. To our knowledge, this is the first biotechnology product "100% made in Palestine", and this action will be a first step towards establishing an industrial biotechnology research platform at Palestine Polytechnic University for the help and benefit of the local society.

ملخص بالعربية

استنساخ الجين المسؤول عن انتاج الأنزيم الرابط للحمض النووي منزوع الأكسجين

علياء المناصرة

إن اكتشاف مجموعة الأنزيمات المهمة التي تسمح بإتمام عملية الاستنساخ للجينات المهمة، قد ساهم في احراز تقدم كبير في مجالي الأحياء الجزيئية والتكنولوجيا الحيوية خلال الخمسين سنة الماضية. وأحد أهم هذه الأنزيمات التي تم الاعتراف بأهمية دورها في تطور التقنية الحيوية هو الأنزيم الرابط الذي يعمل على إعادة بناء الرابطة بين طرفي الحمض النووي منقوص الأكسجين، في تفاعل متعدد الخطوات يعتمد على الطاقة، مما يسمح بإعادة ربط قطع الحمض النووي منقوص الأكسجين سواء أكانت هذه القطع من نفس المصدر او من مصادر مختلفة.

نظراً لدور هذا الأنزيم في مجال التكنولوجيا الحيوية الحديثة، فإن الطلب عليه في ازدياد وذلك لأنه يسمح بربط قطعة الحمض النووي منقوص الأكسجين مع حامل "بلازميد" كخطوة في عملية الاستنساخ.

يهدف هذا البحث إلى عزل الفيروس البكتيري الذي يحتوي على الجين المسؤول عن إنتاج هذا الأنزيم، واستنساخه، وترجمته إلى بروتين. وقد تم عزل الفيروس المسؤول عن إنتاجه من عينات من مياه الصرف الصحي والتي تعتبر البيئة المثالية لتواجد هذا النوع من الفيروسات، ثم تم استنساخ الجين بنجاح ليكون أول منتج في مجال التكنولوجيا الحيوية في فلسطين.

DECLARATION

I declare that the Master Thesis entitled "**Cloning and expression of a bacteriophage DNA ligase** " is my own original work, and hereby certify that unless stated, all work contained within this thesis is my own independent research and has not been submitted for the award of any other degree at any institution, except where due acknowledgment is made in the text.

Alia' Mousa AL-Manasra

Date: 28th July 2011

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Alia' Mousa AL-Manasra

Date: 28th July 2011

DEDICATION

To my dear parents "a great man and a virtuous woman" who gave me all their support until this moment.

To the eternal soul; my sister Amal who shared me all my life moments until 13\11\2007.

To the whole family; my brothers, sisters, uncles, and aunts.

To my friends all over the world.

To all these people; I dedicate my modest work.

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ABBREVIATIONS

a.a	Amino acid
AMP	adenosine monophosphate
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BRC	Biotechnology Research Center
$CaCl_2$	Calcium Chloride
°C	Celsius degree
DBD	DNA-Binding Domain
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotides triphosphate
dsDNA	Double stranded DNA
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic Acid
EMB	Eosin Methylene Blue
EtBr	ethidium bromide
g	Gram
His	Histidine
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Kb	Kilo base
KDa	Kilodalton
LB	Luria broth
M	Molarity
min	Minute
µl	Microliter
ml	Milliliter
mM	Millimolar
mw	Molecular weight
NaCl	Sodium chloride
NAD+	Nicotinamide adenine dinucleotide
NCBI	National Center for Biotechnology Information
ng	Nanogram
nm	Nanometer

N Tase	Nucleotidyl transferase
OB	Oligomer- Binding
OD	Optical density
OH	Hydroxyl
O/N	Overnight
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PO4	Phosphate
PPi	Pyrophosphate
RNA	Ribonucleic acid
rpm	Round per minute
RT	Room Temperature
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	SDS- Polyacrylamide Gel Electrophoresis
sec	second
ssDNA	Single stranded DNA
TBE	Tris base EDTA buffer
TEMED	Tetramethylethylenediamine
T4Dnl	T4 DNA ligase
UV	Ultraviolet
V	Volt
v/v	Volume by volume
w/v	Weight by volume

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CHAPTER ONE

INTRODUCTION

Background

Major advances in biotechnology and molecular biology are attributed to the discovery of certain enzymes such as DNA ligase that allows molecular cloning of important genes.

Polynucleotide ligases stick together 3' OH and 5' PO4 ends in DNA and RNA molecules; using a ligation multistep reaction that involves utilization of an AMP molecule to be covalently attached to both ligase and polynucleotide substrate, respectively (Pascal, 2008).

Polynucleotide ligases are universal enzymes that present in almost all living organisms; they are housekeeping enzymes that are required for survival functions and cellular process related to breaks correction in the nucleic acid backbone structure by joining the 3' hydroxyl and 5' phosphoryl group ends and forming phosphodiester bonds. They have essential roles in DNA replication, repair and recombination (Rossi et al. 1997).

Polynucleotide ligases are categorized into two types according to the source of their cofactor; they are either ATP- or NAD+-dependent enzymes (Pascal, 2008). The organisms that utilize ATP as a cofactor for DNA ligase are Archaea, viruses and Eukarya, while Eubacteria depend on NAD+-dependent DNA ligase to perform ligation mechanism (Wilkinson et al., 2001).

Despite that both of polynucleotide DNA and RNA ligases have many differences in their structural domains, generally they utilize multi-domain construction to perform the multi-

step ligation reaction mechanism. All polynucleotide ligases share a common feature and critical building block in their structural construction which is the nucleotidyltransferase domain (NTase). This domain is located in a specific manner with N- and C-terminal appendages to maintain the overall ligation reaction and to provide substrate specificity according to unique DNA/RNA binding properties (Pascal, 2008).

Both ATP and NAD+ -dependent DNA ligases depend on different accessory domains to perform the formation of the first step of the ligation reaction (ligase-AMP) intermediate. ATP-dependent DNA ligases utilize Oligomer-Binding (OB) domain that is located near the C-terminus of NTase, while NAD+-dependent DNA ligases utilize *Ia* domain which is an N-terminal extension of the NTase to perform this function. The RNA ligases, on the other hand, depend on their NTase domain and don't use such accessory domains to perform (ligase- AMP) intermediate (Pascal, 2008). In addition to NTase and OB domains, ATP-dependent DNA ligases contain also DNA-Binding Domain (DBD) that maintains the substrate (DNA) conformational change needed for ligation activity (Pascal, 2008).

1.1 T4 DNA Ligase (T4Dnl)

T4 bacteriophage is a double stranded DNA virus of about 169 Kb encoding about 300 genes that produce the virus proteins (Bullard and Bowater, 2006). One of the most important proteins for virus metabolism, genome replication, recombination and repair is T4Dnl which catalysis phosphodiester bond formation between 5' PO4 and opposing 3' OH groups in a DNA molecule. It is encoded by gene 30 (*gp30*), with full length of 487 amino acids and molecular weight (MW) of 55.3 kDa (Bullard and Bowater, 2006, Armstrong et al., 1983, Yuan et al., 2007).

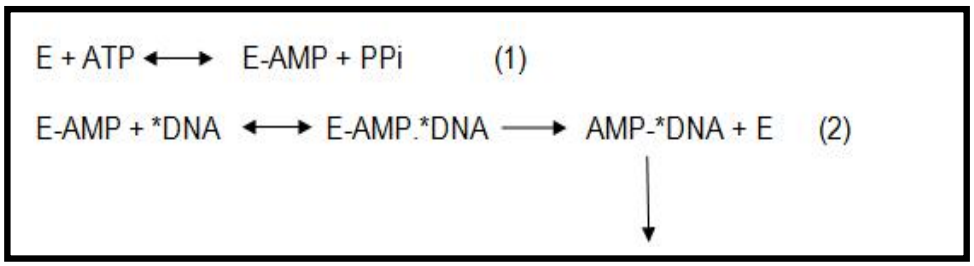
DNA ligase from T4 bacteriophage is the most versatile and commonly used in molecular cloning and recombinant DNA technology (Wilkinson et al., 2001). It is an essential enzyme for DNA replication, repair and recombination. It creates phosphodiester bonds between opposing 3' OH and 5' PO4 ends (Rossi et al., 1997) and is also able to ligate sticky and blunt ends connecting the two dsDNAs and performing nick ligation by sealing breaks between ssDNAs (Cherepanov and Vries, 2003).

1.2 Ligation mechanism

Ligation reaction is energy dependent and consists of three successive steps, which involves two covalent reaction intermediates (Figure 1). In the 1st step; ligase is activated by covalent attachment between α-phosphate of AMP molecule and the enzyme creating a ligase–AMP intermediate and releasing inorganic pyrophosphate (PPi).

In the 2nd step; the AMP group is transferred from ligase to the 5' end phosphate group of the DNA molecule creating an AMP-DNA intermediate. In the 3rd step; the hydroxyl group on the 3' end of the break in the substrate attacks the phosphate on the 5' end of the opposing nucleic acid strand creating continuous backbone structure of the DNA molecule and releasing a free AMP (Rossi et al., 1997, Wilkinson et al., 2001, Cherepanov and Vries, 2003, Johnson and O'Donnell, 2005, Bullard and Bowater, 2006, Yuan et al., 2007, Pascal, 2008).

(A)



(B)

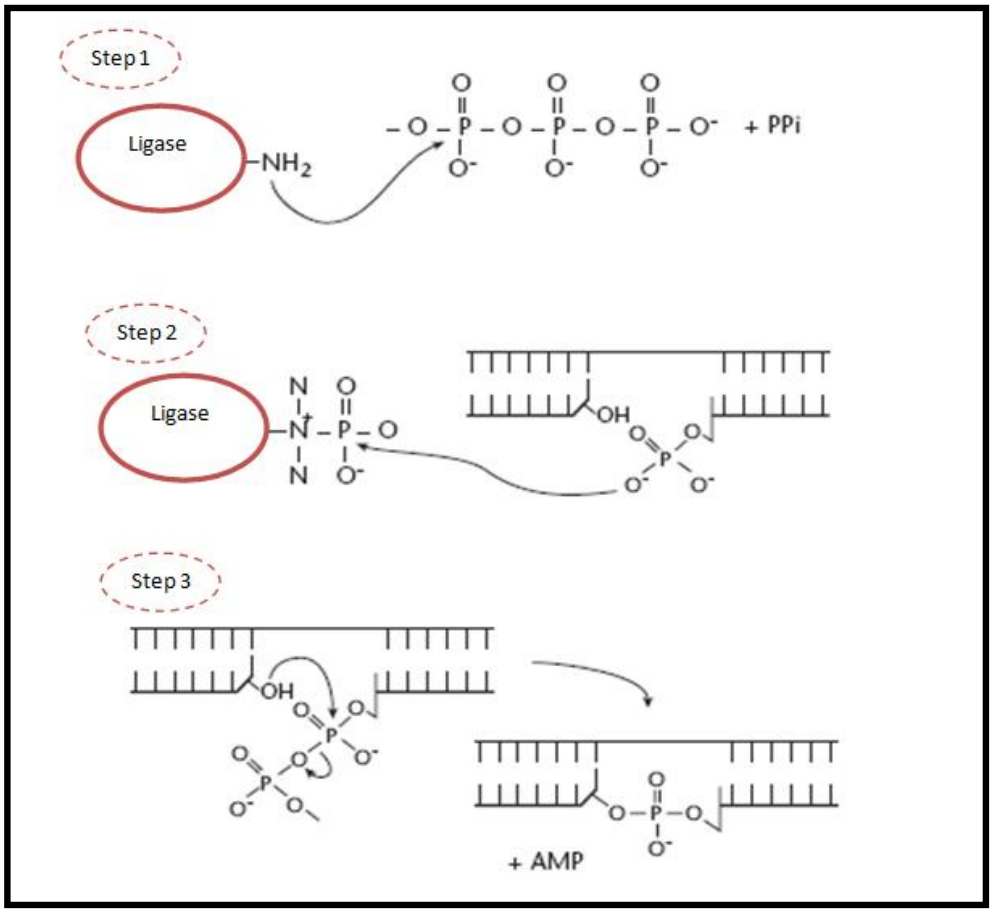


Figure 1. Steps of DNA ligation mechanism

A. Symbolized ligation reaction showing energy requirement.

B. The three major steps in the ligation reaction. In the 1st step; the enzyme is activated by AMP creating E-AMP intermediate and releasing PPi. In the 2nd step; the AMP is transferred to 5' end of the DNA creating AMP-DNA intermediate. In the 3rd step; the 3' end of the DNA attacks the 5' end and seals the nick

One molecule of ATP is needed to be hydrolyzed into AMP and PPi in order to form one phosphodiester bond between dsDNA (Cherepanov and Vries, 2003).

It has been noticed that the most important components of the ligation reaction in order to be performed on time are the presence of 5' phosphorylated end and the abundance of ATP that triggers mechanism initiation by the activation of the enzyme during AMP-Ligase intermediate formation, and the presence of ligase itself (Yuan et al., 2007).

1.3 Work Significance

DNA ligase is one of the most essential enzymes that are used for molecular cloning and recombinant DNA technology.Recombinant DNA technology facilitates protein production by overcoming the problems of source availability of certain proteins such as cytokines, overcoming the problems of source safety such as naturally produced proteins in pathogenic and dangerous species, increasing the amount of produced proteins by enhancing the level of expression, and inserting specific modifications to amino acid sequence of a targeted protein.

The expression of recombinant proteins can be classified either as homologous if the protein is expressed in the same cell that is naturally occurring in or heterologous protein production if the expression is performed in a cell that doesn't naturally occur in it (Walsh, 2002).

The unprecedented recent advances in molecular biology and biotechnology are attributed to the discovery of enzymes that allow molecular cloning of genes that encode important traits such as *Taq* polymerase, DNA ligase, and restriction enzymes. The most commonly used ligase for biotechnological applications of gene cloning is T4 DNA ligase.

The Biotechnology Research Center (BRC) at Palestine Polytechnic University has been founded on the premise of providing cutting-edge biotechnology and molecular biology tools to be used in training and research for the benefit of the local population.

There are some challenges associated with this objective, such as the availability and the expenses of material needed to carry out the training and research in biotechnology. All molecular biology research taking place at BRC requires recombinant biology enzymes, such as T4 DNA ligase.

This project has emerged to support a trend of establishing a research aimed at providing such critical material for biotechnology locally and in a cost-effective manner.

The objectives and outcomes that this project will achieve are:
1) it will provide T4 DNA ligase locally so it can be used by trainees and researchers at BRC with no limitations.

2. It will be the first step towards the production of more health-related, therapeutical, industrial, and agricultural biotechnology products.

3) It will save much needed expenses currently used to purchase this material from other suppliers.

4) It will provide training for graduate students in molecular cloning and microbiology

5) And in the long term, it will make this material available to other researchers and institutions which will provide some income to BRC to cover some of the cost of providing this material.

6) It will determine if the sequence of the ligase gene isolated from local bacteriophage isolates differ from published sequences of European and North American T4 strains.

1.4 Similar Work

1) Functional Characterization of the T4 DNA Ligase: a New Insight into the Mechanism of Action (Rossi et al., 1997).

In this research article, the authors studied DNA ligation mechanism and provided functional characterization of T4 DNA ligase. They compared the enzyme activity between wild type and four mutated enzymes that were purified by His-tag affinity chromatography. The T4 DNA ligase gene was PCR amplified with added *BamHI* restriction sites to BLI forward primer and BLS reverse primer, then it was cloned into the pCR™II TA vector, and expressed in pTreHis-A expression vector.

The recombinant proteins were cloned in *DH5α* strain of *E.coli*, and then they were purified and monitored by silver staining on SDS-PAGE gel and the quality test assays were performed.

They introduced deletion mutations at His 80 – N-terminal and His 57 –C-terminal, they found that these deletion mutants still perform nick joining activity, since these mutations do not affect the catalytic domain of the enzyme, but they fail to perform blunt end ligation since N and C terminals are essential portions to form a stable enzyme-DNA complex.

2) Production of Recombinant Enzymes of Wide Use for Research (Manzur et al., 2006).

This paper discussed the production of two important recombinant enzymes for biotechnological applications, reverse transcriptase MMLV and *Taq* DNA polymerase which they expressed in pTTQ18 vector.

Taq DNA polymerase was purified by heating dependent method, while MMLV reverse transcriptase was purified by lysozyme treatment and sonication method, then they were monitored on SDS-PAGE gel, and activity tests were performed.

They developed a purification protocol for *Taq* polymerase depending on its high temperature resistance, and compare its efficiency by the one wich is resulted from published protocols that depends on polymerase solubility and purification by precipitation with NH_4SO_4.

In addition, the authors compared results of their purification protocol MMLV retrotranscriptase extracted from inclusion body fraction, with other protocols depending on soluble fraction. They found their method to be more efficient and suitable for the purification of such proteins associated with inclusion bodies, which negatively affect the quality of purified products.

CHAPTER TWO

Goal and Objectives

2.1 Overall Goal

The overall goal of this project is to clone the gene encoding the enzyme DNA ligase from *DH5a*-specific bacteriophage.

2.2 Specific Objectives

1) To isolate a bacterial strain *DH5a* -specific bacteriophage
2) To isolate, clone and express a bacteriophage DNA ligase gene
3) To detect any sequence differences between our local isolated DNA ligase gene and the published sequences in GenBank database

CHAPTER THREE

MATERIALS AND METHODS

3.1 Phage Preparation

Wastewater samples (5 ml each) were collected from an area in south Hebron with open undeveloped sewage system and used to infect 0.5 ml overnight (O/N) culture of *DH5α.* The mixture was allowed to incubate O/N at 37 °C with continuous shaking at 200 rpm. In the morning of the next day, 1.0 ml of the viral culture was transferred to sterile microfuge tube, one drop of chloroform (Frutarom/ 5551020) was added and mixed by inversion, then it was centrifuged at 12,000 rpm for 2 min. The supernatant was transferred to a new microfuge tube before it was centrifuged again.

The supernatant was filtrated by 5cc syringe (A0.22micron) filter disc and the filtrate (enriched phage prep) either stored at 4 °C or used to lyze bacterial cells grown on agar plates a night before performing plaque assay. The resulted clear zones represented phage particles. These zones were cut and stored at -80 °C as stab culture for future use.

To prepare culture for genomic DNA extraction, a 5 ml O/N culture of *DH5α* was infected with stab culture of bacteriophage, and incubated under continuous shaking (200 rpm) at 37 °C. Following incubation, 1.0 ml of the O/N viral culture was transferred to sterile microfuge tube, one drop of chloroform was added and mixed by inversion before centrifugation at 12,000 rpm for 2 min. The supernatant was transferred to a new tube and centrifuged again to isolate the viral particles. The supernatant was filtrated by 5cc syringe (A0.22micron) filter disc before it was stored at 4 °C. Plaque assays were

performed as a control. This means that when a bacteriophage is added to a bacterial plate, it will infect and lyse bacterial cells in the place of addition to produce a clear area as a result of infection. This clear zone is called a plaque (Havelaar and Hogeboom, 1983).

3.2 Isolation of a *DH5α* Specific T4 Bacteriophage

Two different agar Petri dishes were plated one with native *E.coli* which was isolated by using EMB media (Oxoid, 689340) (Figure 2), the other with *DH5α. After* 15 minutes 10 µl of phage prep were pipetted at the center of each plate, the plates were incubated O/N at 37 °C.

Native *E.coli* cells were isolated by plating 100 µl of wastewater sample on EMB differentiated media. Following O\N incubation at 37 °C *E.coli* growth appeared as a green metallic sheen (Venkateswaran et al., 1996). In the following day subculture from the green colonies was made in order to have a pure *E.coli* from other species that could be present in wastewater.

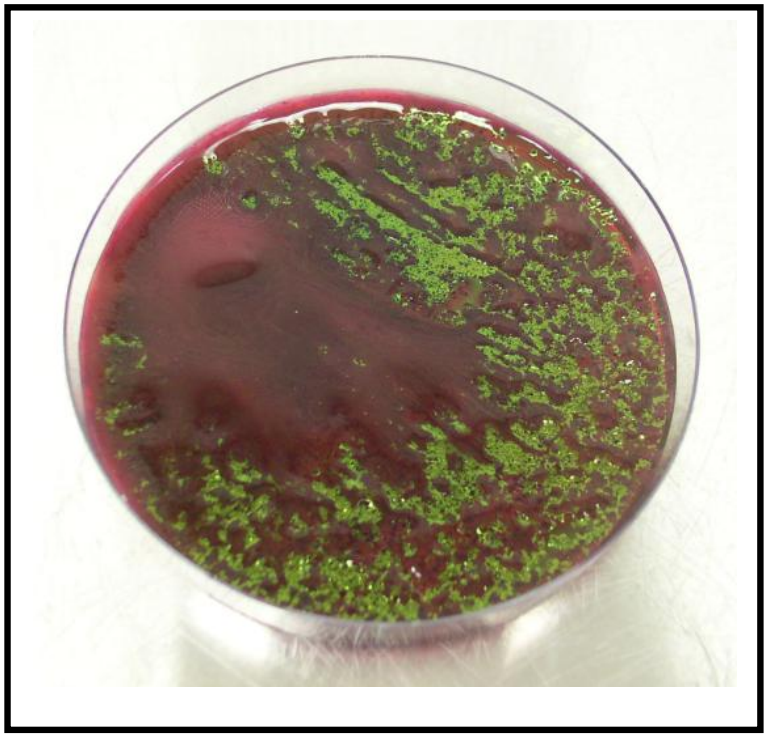


Figure 2. Green metallic sheen of *E.coli*

***E.coli* appears on EMB differentiated media as a green metallic sheen**

Two markers were checked by standard PCR as shown later to confirm whether the isolated bacteriophage is indeed a T4 bacteriophage.

The markers were; 1) the capsid protein *gp20* (Comeau and Krish, 2008), and 2) the *segF*, a site specific DNA endonuclease encoded by gene 69 (Belle et al., 2001).

3.3 T4 Bacteriophage DNA Collection and Extraction

One ml of viral extract was added to 100 ml of O/N incubated *DH5α* culture. The new mixture was incubated for 6 hours at 37 °C before centrifugation at 3600 rpm for 10 min. The supernatant decanted and the pellet stored at -20 °C. The DNA was extracted by using EZ-DNA kit (Genomic DNA isolation Kit) purchased from Biological Industries, with the following modifications. The addition of 1.0 ml of EZ reagent to the pelleted cells before they were lysed by repetitive pipetting to break cells. The samples were then mixed at 60 °C for 60 min and centrifuged at 12,000 rpm for 10 min at room temperature (RT). The supernatant was then transferred to a new microfuge tube. For DNA precipitation; 1.0 ml of absolute ethanol was added/1.0 ml of EZ reagent, the sample was mixed by inverting the tube 10 times and stored at RT for 3 min before it was centrifuged at 6,000 rpm for 5 min. For DNA washing; the pellet washed twice with 1.0 ml of 95% ethanol, the tube was inverted 10 times and then centrifuged at 1,000 rpm for 1 min to allow DNA to settle down. The ethanol removed and the pellet dried for 5 min. The pellet was then dissolved in 100 ml ultra pure water and DNA stored at -20 °C for subsequent use.

3.4 Amplification and Visualization of a T4 DNA Ligase Gene

To amplify the DNA ligase gene, PerlPrimer software was used to design primers based on the T4 DNA ligase sequence accession number X00039 as follows:

T4 F a 5`-GGGGGATCCTAAAGTTAGAACCACGTACCAC-3`

T4 R a 5` GGGCTGCAGGCTTTCATAGACCAGTTACCT 3`

The gene was amplified by PCR using thermostable *Taq* DNA polymerase (Hy Labs/ HTD0078). The reaction mixture consisted of ultra pure water, 10X reaction buffer (Hy Labs/ HTD0078), MgSO4 (Hy Labs/ HTD0078), dNTPs (Sigma/ DNTp10-1KT), forward and reverse primers, and the template DNA (107 ng/µl). PCR amplification was carried out with a thermal cycler under the following conditions: initial denaturation at 95 °C for 2 min, 25 cycles of 95 °C for 30 sec, 54 °C for 30 sec, 72 °C for 2 min, followed by a final extension at 72 °C for 10 min.

Amplified PCR reaction (25 µl) was mixed with 2 ul 6X loading buffer (0.25% (w/v) bromophenol blue (Fluka/ 417639/1), 0.25% (w/v) xylene cyanol FF (Amresco/ 1897B066), 30% glycerol (Amresco/ 0176B017) (Sambrook and Russel, 3, 2001), and then loaded onto a 1% (w/v) agarose (Seakem/ 5080021) gel containing 1X TBE buffer (1L of 5X stock contained 54 g Tris-base (Promega/ H5131), 27.5 g boric acid (Sigma/ 078k0037), 20 ml of 0.5 M EDTA (Alfa aesar/ 10122546), pH 8.0) (Sambrook and Russel, 3, 2001) stained with ethidium bromide (EtBr) (Hy Labs). Gel electrophoresis was carried out in 1X TBE buffer at 90 V. Ultraviolet light emitted from a transilluminator was used to visualize the band corresponding to the expected size of the gene.

3.5 Band Purification from Gel

The DNA was purified from the gel using the AccuPrep® Gel Purification Kit (Bioneer, K-3035) with minor modifications. The DNA fragment was excised from the gel and placed into a microfuge tube. Three volumes of buffer 1 (Gel Binding Buffer) were added to 1 volume of gel, followed by incubation at 60 °C for 10 min with vortexing every 2 min for complete dissolving. Ten µl of 3M sodium acetate (pH 5.0) was added and mixed

until the color became yellow. The mixture was transferred to DNA binding column tube and centrifuged for 1 min at 13,000 rpm, and then DNA binding column tube was re-assembled with a 2 ml collection tube. 500 µl of buffer 2 (washing buffer) was added to the DNA binding column and it was centrifuged for 1 min at 13,000 rpm (this step was repeated), followed by additional centrifugation at 13,000 rpm for 1 min. DNA binding column was transferred to new microfuge tube, 30 µl of ultra pure water was added to the center of the DNA binding column, followed by waiting for 5 min at RT. The DNA fragment was eluted by centrifugation at 13,000 rpm for 1 min, and then it was stored at -20 °C for future work.

3.6 Preparation of competent cells

One hundred ml of LB (1 L of LB contained 10 g trypton (Neogen/120- 125A), 10 g NaCl (Frutarom/2355534710), 5 g yeast extract (Neogen/120- 185A) was infected with 10 ml O/N culture of *DH5α* and incubated at 37 °C for about 3 hours until cell density reached 0.6 OD at 600 nm. Fifty ml of the culture was transferred to conical tube and centrifuged at 2, 000 rpm for 10 min. The supernatant decanted and the pellet re-suspended with tip cut pipet in 10 ml of ice cold 0.1M $CaCl_2$ before centrifugation at 2,000 rpm for 10 min. The supernatant decanted and the pellet re-suspended in 1.0 ml of ice cold 0.1M $CaCl_2$. Competent cell aliquoted in 50 µl volumes in 1.5 ml Eppendorf tubes and stored at -80 °C for subsequent use.

3.7 Cloning of T4 DNA Ligase Gene

The gene was cloned into the pGEM® -T Easy (Promega/A1360) cloning vector (Figure 2) via a ligation reaction containing 2X ligation buffer, 50 ng pGEM® -T Easy vector,

233 ng of insert (according to a 3:1 insert: vector molar ratio) and 3 Weiss units of T4 DNA ligase, to a final reaction volume of 10 µl. The ligation reaction was overnight incubated at 4 °C, and then they were used for transformation in *DH5α* competent cells, and the rest was stored at -20 °C for future transformation.

Cloning in pGEM® -T Easy depends on blue-white selection technique, it contains two selectable markers; the 1st one is an ampicillin resistant gene, which indicates the positive transformed cells. The 2nd one is a *lacZ* gene which distinguishs between positive and negative cloning (Promega, technical manual). The *lacZ* encodes β-galactosidase that breaks X-gal in the media to produce blue color.

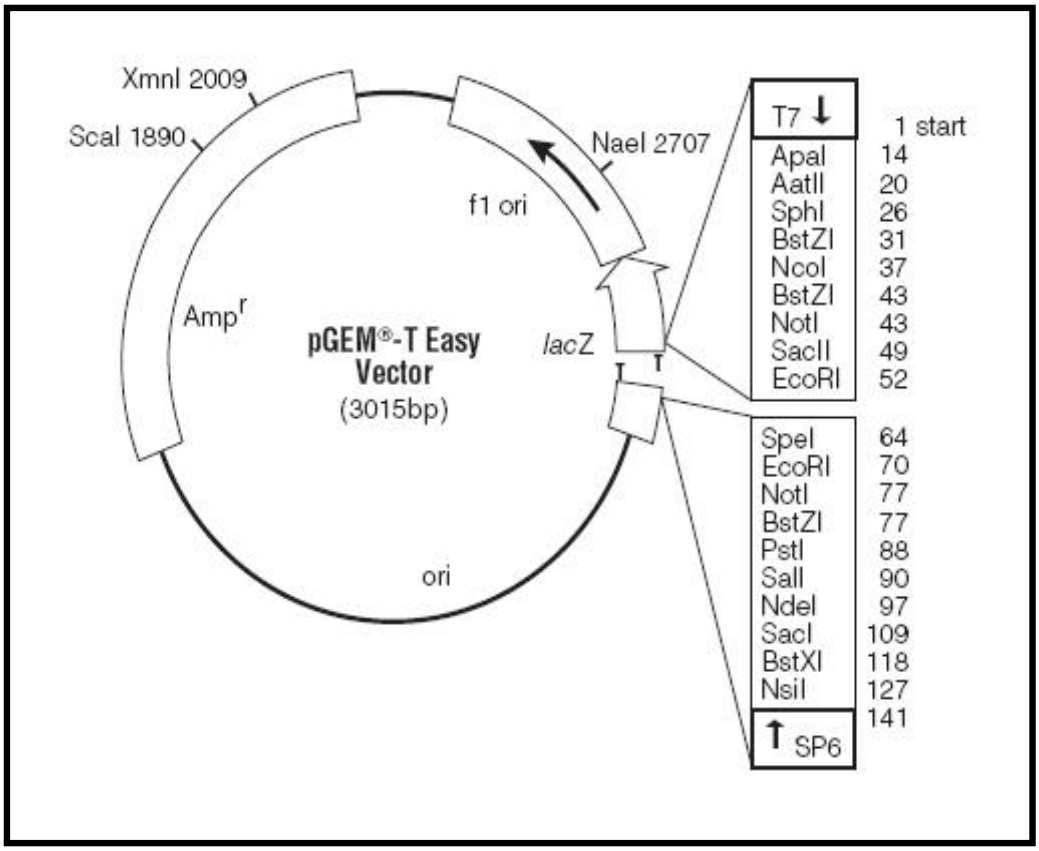


Figure 3. pGEM® -T Easy map

Detailed structural map of pGEM® -T Easy vector. Inserts are cloned by the facilitation of the T overhangs as shown (Promega, technical manual).

3.8 Transformation into *DH5α*

The overnight ligation reaction of DNA ligase gene-pGEM®- T Easy was removed from refrigerator and placed at room temperature for 30 min before starting the transformation.

Fifty µl *DH5α* competent cells were removed from -80 °C deep freezer and placed on ice for 30 min to thaw, at the same time a small flask of LB warmed at 37 °C. Five µl (1- 50 ng) of the ligated DNA were added to the competent cells, mixed gently by tapping and placed on ice for additional 30 min. the mixture of competent cells with ligated DNA heat shocked exactly at 42 °C in a water bath for 45 sec, and then returned to ice for 2 min. Nine hundred and fifty µl of pre-warmed LB were added to the transformed cells, and incubated in water shaker bath at 37 °C, 180 rpm for 6 hours. Two hundred µl of transformed cell culture were plated onto labeled agar plates containing 25 µl of 50 mg/ml ampicillin (Applichem/ 8G005753), 100 µl of Isopropyl β-D-1 Thiogalactopyranoside IPTG (Sigma, 128K4053); for *lacz* induction, and 20 µl of 100mg X-Gal (Applichem/ A4978.0100)/2 ml formamide (Fluka/ 1245742); per each agar plate that contains 25 ml LB. The plates were sealed, placed for 30 min upside up to allow absorption, and then they were incubated overnight at 37 °C.

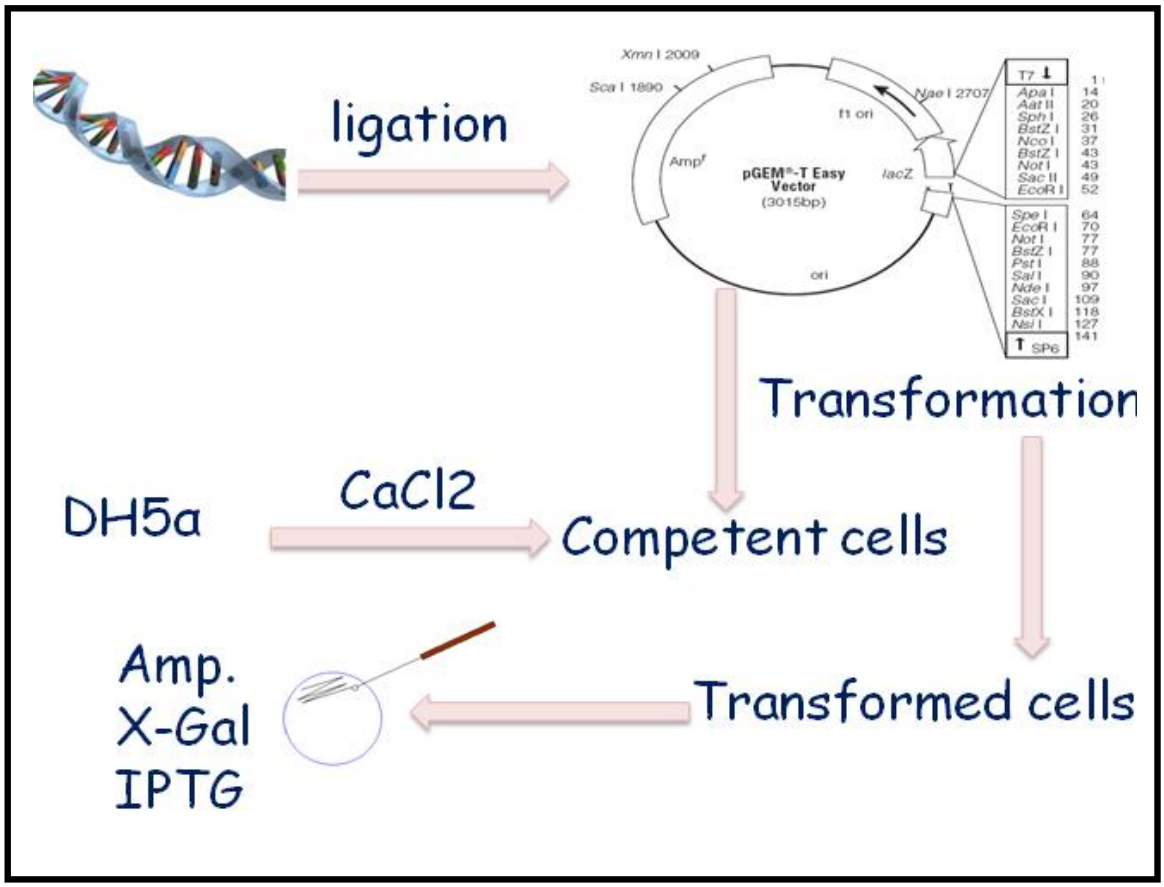


Figure 4. symbolized cloning process in pGEM® -T Easy

The gene was inserted ito pGEM® -T Easy cloning vector, then it was transformed by heat shock in DH5α competent cells, then the transformed cells were grown on a selective media containing ampicillin, X-Gal and ITG.

3.9 Screening and Verification of Positive Clones

Verification was performed at 3 levels:

The 1st level by depending on an antibiotic resistance, media contains ampicillin, only the transformed cells that contain an ampicillin resistance gene will grow.

The 2nd level depends on blue-white selection media containing X-gal that will be broken by β-galactosidase enzyme encoded by *lacZ* gene. If cloning has failed, then the *lacZ* gene is not disrupted and as a result the β-galactosidase enzyme is functional to break x-gal and produce blue-colored colonies, while white colonies indicate positive cloning due to the disruption of *lacz* gene (Figure 5).

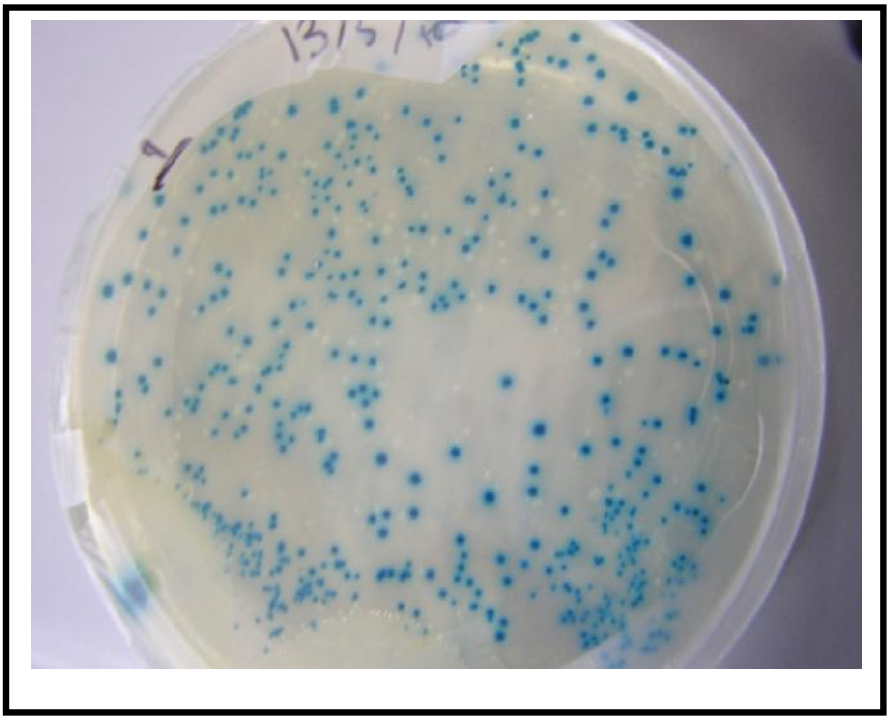


Figure 5. Blue white selection

White colonies are positive clones that means the gene was inserted inside a LacZ gene, and β-galactosidase could'nt perform its function which is breaking X-Gal in the media to produce blue color,while blue colonies are negative which means tha the β-galactosidase is still working.

The 3rd level depends on a PCR technique to confirm that the white colonies grown on agar plates containing ampicillin/IPTG/X-Gal were positive clones for a T4 bacteriophage DNA ligase.

PCR verification was also performed on three samples; colonies, liquid culture and purified plasmid by using the same reaction conditions, mixture, primers and PCR program that were used to amplify a DNA ligase gene from genomic DNA of a

bacteriophage. The amplified gene was visualized by gel electrophoresis, and the resulted specific band was purified from gel as described above for future work.

3.10 Subsequent Cloning of T4 DNA Ligase Gene into Expression Vector

In order to ligate a DNA ligase gene into the expression vector pPROEX HTb (Figure 6), both the purified DNA ligase gene and pPROEXHTb expression vector were double digested with *BamHI* (Biolabs/ R0136S) and *XhoI* (Biolabs/ R0146S) restriction enzymes. The digestion reaction for pPROEX HTb vector consisted of 5 µl of 10X NEBuffer 3:0.5 µl of 100X BSA, up to 1µg plasmid DNA, 1 unit each of *BamHI* and *XhoI*, then the reaction volume was completed with ultra pure water to a final volume of 50 µl. The digestion reaction for a DNA ligase gene was consisted of 5 µl of 10X NEBuffer 3, 0.5 µl of 100X BSA, up to 1µg of a DNA ligase gene, 1 unit each of *BamHI* and *XhoI*, then the reaction volume was completed with ultra pure water to a final volume of 50 µl. The reaction components of each tube were mixed gently and then incubated for 1:15 hour at 37 °C. Then the two reactions were loaded on an agarose gel, in order to terminate the digestion activity of the restriction enzymes. According to their size; the cut bands were purified from gel as described previously and concentration was taken for ligation reaction in the following step. Both of *BamHI* and *XhoI* have only one cut site within the multiple cloning site of pPROEX HTb, and neither cut the open reading frame of the DNA ligase gene.

The digested DNA ligase gene was ligated with the digested pPROEX HTb expression vector via ligation reaction containing 2X ligation buffer, 61 ng pPROEX HTb vector, 46 ng of insert and 3 Weiss units of T4 DNA ligase. Ultra pure water added to a final

reaction volume of 10 µl. The ligation reaction incubated overnight at 4 °C before used for transformation into *DH5α* competent cells. The remaining ligation reaction was stored at -20 °C for future transformation.

In the following day; pPROEXHTb containing a DNA ligase gene was transformed into *DH5α* bacterial cells, as mentioned in the protocol above, with the transformed cell culture plated onto labeled agar plates containing only ampicillin as a selectable marker.

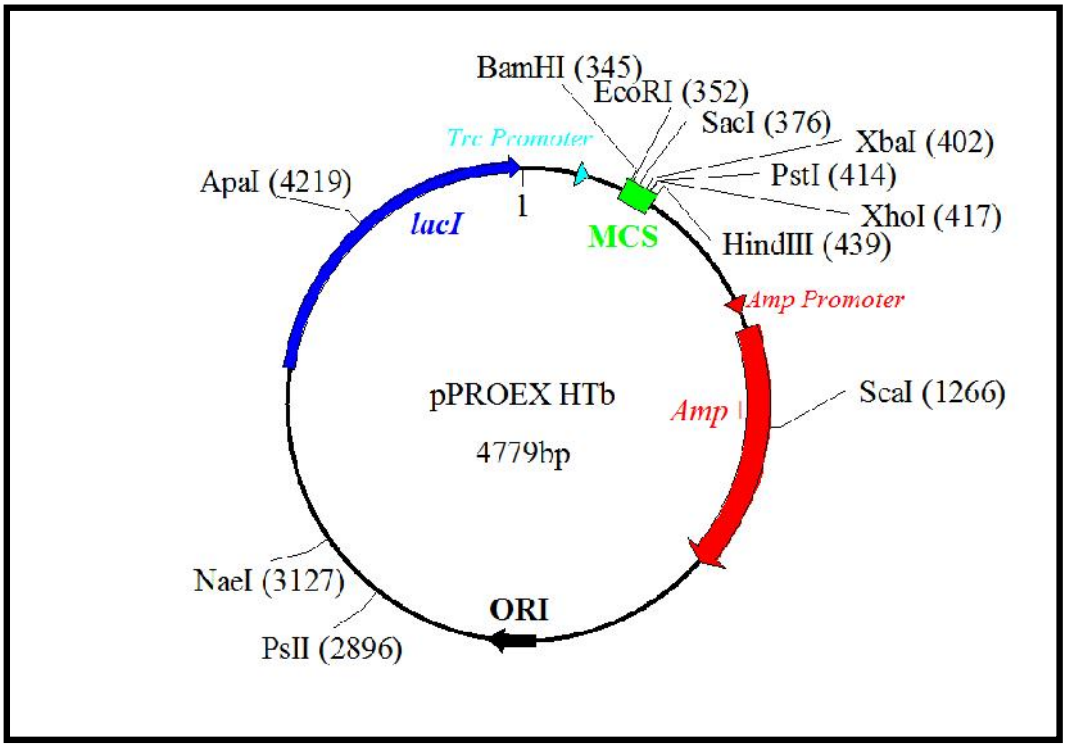


Figure 6. Detailed structural map of pPROEX HTb vector
This expression vector contains His tag to facilitate protein purification, also it contains Amp resistant gene, but lacks blue/white selectable markers.

3.11 Screening and Verification of Positive Clones

Two verification methods were used to confirm that ampicillin resistant colonies were grown on agar plates containing ampicillin were positive clones for a bacteriophage DNA ligase.

1) PCR verification was performed at three levels; on colonies, liquid culture and purified plasmid by using the same reaction conditions, mixture, primers and PCR program that were used to amplify a DNA ligase gene from genomic DNA of a bacteriophage. The amplified gene was visualized by gel electrophoresis, and the resulted specific band was purified from gel and prepared for next verification step.

2) Sequencing the purified band by using 3 sets of primers which was performed as a service in Hereditary Research Laboratory/ Life Science Department/ Bethlehem University (Bethlehem, Palestine).

Sequence results of a bacteriophage DNA ligase were compared using Kalign (http://www.ebi.ac.uk/Tools) with other cds sequence that was published in the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/). The sequence was translated using expasy translation tool (http://www.expasy.ch/tools/dna.html), then the resulted protein sequence was compared with the published one at NCBI using protein Basic Local Alignment

Search Tool (BLAST) to determine the different amino acids between both sequences.

3.12 His- tag Protein Purification

Five hundred ml LB containing 500 µl of ampicillin (50mg/ml) were infected with 5 µl transformed bacterial cells (the transformed cells were stored as glycerol stock which was prepared by combining half ml of cells with half ml of glycerol to protect cells when it was stored at – 80 ° C) with pPROEXHTb before incubation at 37 °C until cell density reached 0.6 at OD 600 nm. Then the culture was induced by the addition of 500 µl 100 mM IPTG to a final concentration of 1 mM before overnight incubation at 37 °C.

The cell culture was centrifuged at 8000 rpm, 4 °C for 10 min, and the supernatant was discharged while the cell pellet resuspended with 50 ml Phosphate Buffer Saline (PBS) before it was centrifuged one more time at 8000 rpm, 4 °C for 10 min. The supernatant discharged and the cell pellet stored at -80 °C until the following day.

The pellet of each tube resuspended in 1 ml lysis buffer containing PMSF (Sigma/ P7620), lysozyme (Fluka/ 1399916) and 10 mM imidazole (Fluka/ 1403776), then centrifuged for 10 min, at 13000 rpm and 4 °C to separate supernatant from pellet which was kept at – 20 °C to be checked on SDS-PAGE-.

Ligase purification was carried out using affinity chromatography using 50% Ni-NTA Superflow (Sigma/ H 0537) columns.

One ml of Ni Nickel resin washed with washing buffer that contains 20 mM of imidazole to remove non specific protein. Following resin settlement, the buffer was removed, and

the resin mixed with supernatant (i.e., total protein extract) that was separated from the previous step. The mixture was incubated with shacking at room temperature for 60 min to allow nickel resin binding with the His tag of the recombinant ligase.

The column was prepared with the mixture poured in the column and the flow through collected for leakage checkup on SDS-PAGE. The beads were washed three times with 1 ml washing buffer and the three washing samples were kept to be checked later on SDS-PAGE.

Recombinant protein was eluted four times with 500 µl elution buffer that contains high concentration of imidazole (250 mM), the elution samples were kept to be checked on SDS- PAGE for protein purity.

All the elution process occurred on ice, and all the samples were stored at – 20 °C until they either loaded on SDS- PAGE, or their activity checked by quality control tests.

3.13 SDS-PAGE Polyacrylamide Gel Electrophoresis

The protein samples that were collected previously, including pellet, flow through, washing (W1, W2, W3) and elution (E1, E2, E3, E4) were loaded on 10% SDS-PAGE gel for separation.

The glass plates of gel system were cleaned and assembled, after determination the volume of resolving and stacking gels.

Resolving gel solution (10%, 20 ml) was prepared by combining 7.8 ml ultra pure water, 6.7 ml of 30% acrylamide mix (Alfa asear/ A17157), 5 ml of 1.5 M Tris (pH 8.8), 200 µl of 10% SDS (Biological industries/ 747261), 300 µl of 10% ammonium persulfate

(APS), and 10 µl Tetramethylethylenediamine (TEMED) (Sigma Aldrich/ T2,250-0). Resolving gel solution casted into the gap between the glass plates by using a plastic dropper, 1 ml of water was added to the gel surface in order to keep its moisture, the gel was placed vertically at room temperature for about 60 min until it was polymerized.

Stacking gel solution (5%, 6 ml) was prepared by combining 4.1 ml ultra pure water, 1 ml of 30% acrylamide mix, 750 µl of 1.5 M Tris (pH 8.8), 60 µl of 10% SDS, 60 µl of 10% APS, and 6 µl TEMED. Water on the surface was completely sucked by filter paper, and then stacking gel solution was poured directly on the surface of resolving gel. Immediately a clean Teflon comb was inserted onto the stacking gel solution without making air bubbles. Place it for additional 30 min at room temperature.

After polymerization; the samples were prepared by mixing 20 µl of each with 10 µl of 2X SDS Gel-loading buffer that was composed of 100 mM Tris- Cl (pH 6.8), 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol and 200 mM β-mercaptoethanol (Sigma/ 125K0165). The samples were heated at 100 °C for 3 min to denature a protein. Gel system was assembled in their reservoir, the Teflon comb was removed carefully, running buffer that contained 25 mM Tris base, 250 mM NaCl and 0.1% SDS was added to the gel in the reservoir. Then the samples were loaded at the bottom of the wells in a known order.

Electrophoresis apparatus was attached to an electric power supply with 150 volts for about 2 hours until it was separated to the end. Then the power supply was turned off and the gel was removed carefully, gel orientation was marked by end cutting.

The gel was stained by Coomassie Brilliant Blue (Applichem/ A3480,0010) prepared by dissolving 0.25 g of Coomassie Brilliant Blue powder in 100 ml of methanol (Frutarom/ 5552390) - acetic acid (Frutarom/ 5550020) solution (5 methanol: 4 water: 1glacial acetic acid). The gel immersed in the staining solution and placed on a rotating plate for an hour at room temperature.

Remaining stain solution was removed for future uses, and the gel soaked in fixing solution (1 glacial acetic acid: 2 methanol: 7 water). This process was repeated three times, the 1^{st} and the 2^{nd} for 15 min for each and the 3^{rd} it was occurred overnight on a rotating plate.

In the following day the gel was removed from fixing buffer, visualized by naked eyes and documented by photography before the gel was finally dried and stored.

CHAPTER FOUR

RESULTS

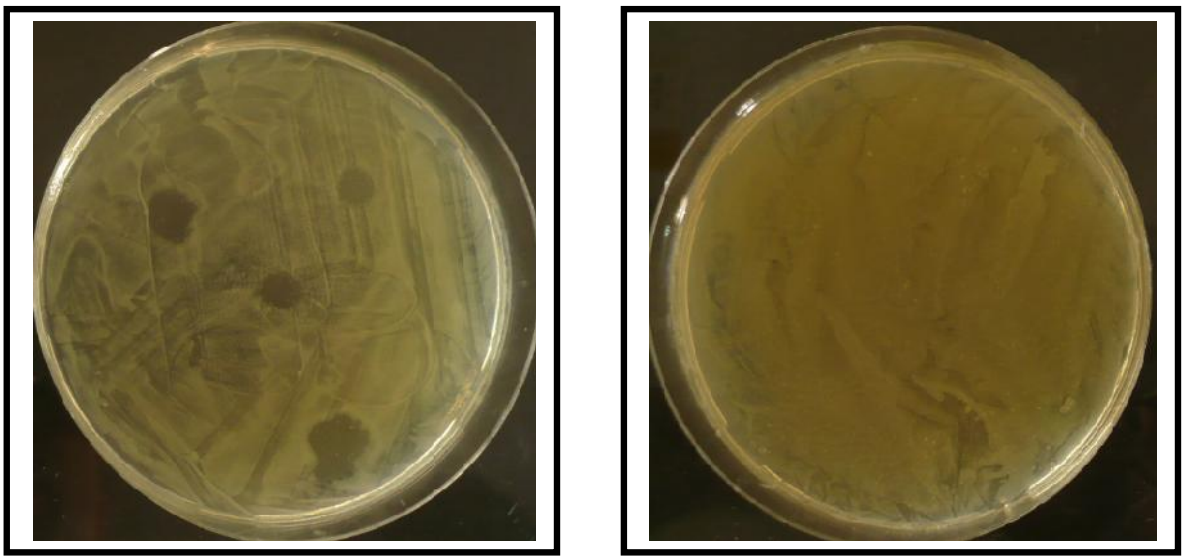
4.1 A strain specific bacteriophage was isolated

A strain-specific bacteriophage was isolated from enriched bacteriophage culture by performing the plaque assay (Havelaar and Hogeboom,1983). A *DH5α* culture was infected by phage particles isolated from wastewater collected from Hebron sewage. The *DH5α* is a commercial strain of *E.coli* commonly used in laboratories.

Bacteriophage extract added onto two plates, one of them plated with native *E.coli* and the other containing *DH5α*, and both were incubated overnight at 37°.

Both plates of *E.coli* and *DH5α* were infected by drops of phage extract. Results showed that the *E.coli* was not affected, while the *DH5α* was a specific host for the isolated bacteriophage by forming clear areas in the places of infection as a result of cell lysis by bacteriophage in these zones (Kunapuli, 2010).

The plaques or clear zones appeared as a result of bacteriophage infection in only the *DH5α* plates (Figure 7A), where as plates containing the total *E.coli* were not affected by the addition of a bacteriophage extract as shown in figure 7B.



(A) (B)

Figure 7. Plaque assay

Effect of the isolated bacteriophage on *E. coli*. A. The bacteriophage was able to lyse *DH5α* and generate clear zones representing the areas where the phage particles were pipetted. B. The bacteriophage is unable to lyse the total *E. coli*.

4.2 Amplification of the DNA ligase

A highly specific band matches the size of a bacteriophage DNA ligase gene was pulled out from DNA isolated from lysed bacteriophage-bacteria culture. The gene was amplified by PCR, loaded on agarose gel and visualized by UV light. The gene size is 1464 bp and the amplicon size was 1561bp (Figure 8). The amplified fragment matched the expected size of the ligase gene.

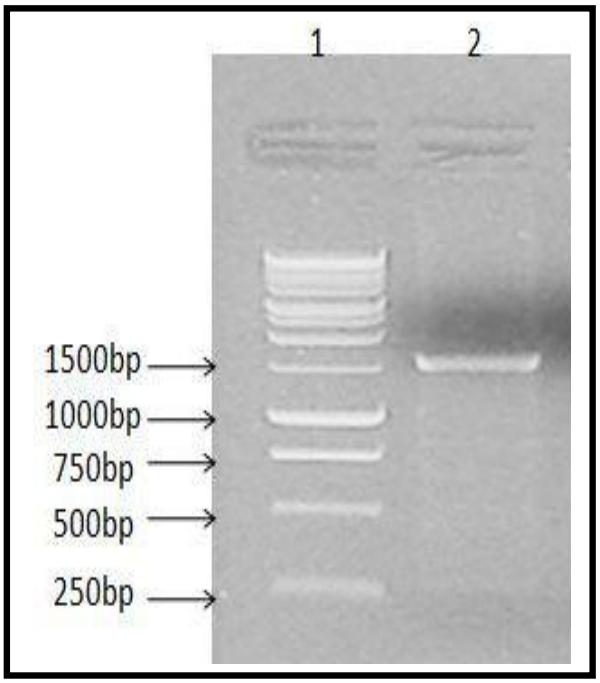


Figure 8. gel photo of the DNA ligase gene.
The ligase gene was amplified and loaded on 1% agarose gel, stained with EtBr and visualized using gel documentation system (right lane). The left lane is showing DNA ladder (Promega/ G5711). The amplified band expected size was 1561 bp and matches 1500 bp on the ladder.

4.3 Successful cloning of the ligase gene in a pGEM® -T Easy

The bacteriophage DNA ligase gene was amplified, purified from agarose gel and successfully cloned into pGEM®-T-Easy cloning vector. Following the ligation reaction and the subsequent transformation of the pGEM®-T-Easy ligase gene into *DH5α* strain as described in the Materials and Methods, the *DH5α* cells were plated onto agar plates containing ampicillin and x-gal for selection as described above. Following the overnight incubation at 37 °C, white and blue colonies appeared, representing positive and negative transformants, respectively (Figure 9). White colonies were chosen for subsequent verification.

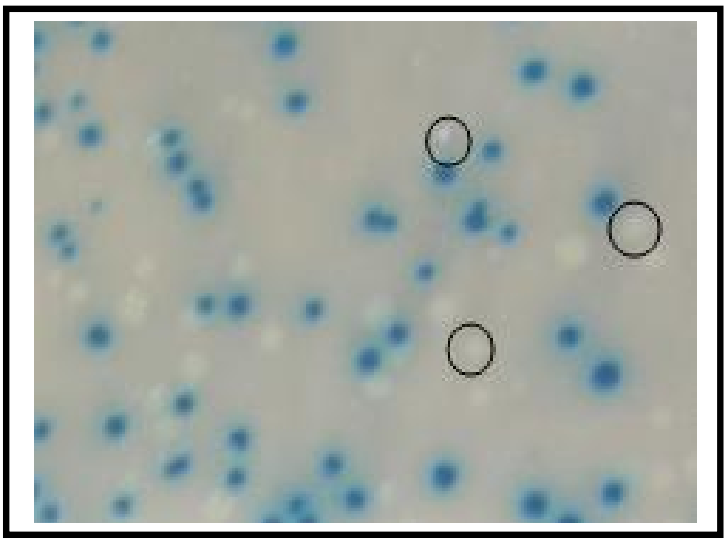


Figure 9. Blue-white selection. Blue colonies are negative clones, while the encircled ones (white colonies) are positive clones and thus they were used for further verification by PCR.

Cloning was verified by PCR that had been performed on colonies, liquid culture and purified pGEM-T-Easy plasmid. The resulted band was highly specific and matched the gene size (Figure 10).

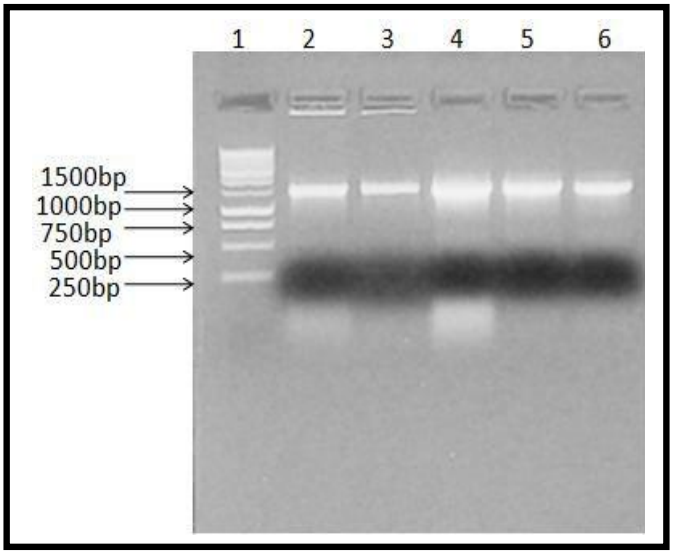


Figure 10. Gel photo of PCR verified positive clones in pGEM® -T Easy The ligase gene was amplified and loaded on 1% agarose gel, stained with EtBr and visualized using gel documentation system (right lane). Lane 1 contains the DNA ladder (Promega/ G5711). Lane 2 shows the amplified gene from bacterial colony. Lane 3 from liquid culture. Lane 4, 5, 6 from purified pGEM® -T Easy plasmid by different ways of purification. Specific band (bacteriophage DNA ligase) that matches the corresponding size in the DNA ladder. The band size is 1561 bp while the gene size is 1464 bp.

4.4 Successful cloning of the ligase gene into a pPROEX HTb expression vector

The ligase gene was successfully cloned into pPROEX HTb expression vector, the cloning was verified by PCR (Figure 11) and sequencing. Cloning into this vector is necessary for gene expression and protein purification. The vector allows affinity purification of the protein as it attached our protein to 6His tag residues

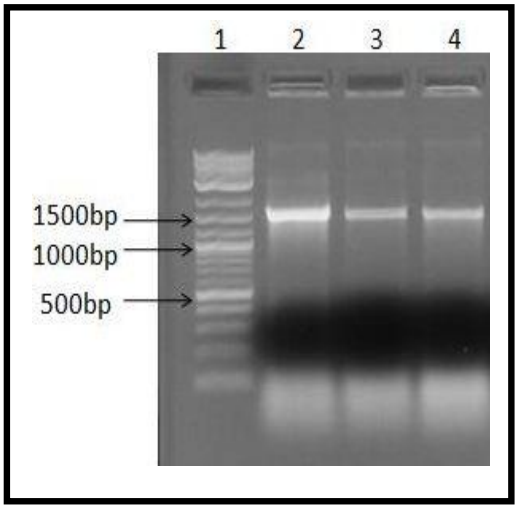


Figure 11. Gel phpto of PCR positive clones in pPROEXH
Lane 1 contains the DNA ladder (Biolabs/ N3200S). Lane 2 shows the amplified gene from colony. Lane 3 from liquid culture. Lane 4 from purified plasmid. Specific band (bacteriophage DNA ligase) that matches the corresponding size in the DNA ladder. The band size is 1561 bp while the gene size is 1464 bp.

4.5 Sequencing

A full sequence of SHPh ligase was obtained by sequencing, SHPh was aligned with X00039.1 cds (T4 ligase gene) sequence that is present at NCBI, the Blast results shows some variations at nucleotide level, most of them are present at the 3rd letter in the codon.. The obtained results were: score 2510 bits (1359), with 0.0 E.value, the identity percentage was 98% (1429/1464), without any gap. Then the nucleotide sequence was compared with the 1st five matches to determine how much they are related (table 1).

```
>lcl|57029 SHPh
Length=1464

 Score = 2510 bits (1359),  Expect = 0.0
 Identities = 1429/1464 (98%), Gaps = 0/1464 (0%)
 Strand=Plus/Plus

Query  1     ATGATTCTTAAAATTCTGAACGAAATAGCATCTATTGGTTCAACTAAACAGAAGCAAGCA  60
             |||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  1     ATGATTCTTAAAATTCTGAACGAAATAGCATCTATTGGTTCAACTAAGCAGAAGCAAGCA  60

Query  61    ATTCTTGAAAAGAATAAAGATAATGAATTGCTTAAACGAGTATATCGTCTGACTTATTCT  120
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Sbjct  61    ATTCTTGAAAAGAATAAAGATAATGAATTGCTTAAACGAGTATATCGTCTGACTTATTCT  120

Query  121   CGTGGGTTACAGTATTATATCAAGAAATGGCCTAAACCTGGTATTGCTACCCAGAGTTTT  180
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Sbjct  121   CGTGGGTTACAGTATTATATCAAGAAATGGCCTAAACCTGGTATTGCTACCCAGAGTTTT  180

Query  181   GGAATGTTGACTCTTACCGATATGCTTGACTTCATTGAATTCACATTAGCTACTCGGAAA  240
             |||||| ||||| |||||||||||||||||||||||||||| ||||||||||||||||
Sbjct  181   GGAATGCTGACTATTACCGATATGCTTGACTTCATTGAATTCACGTTAGCTACTCGGAAA  240

Query  241   TTGACTGGAAATGCAGCAATTGAGGAATTAACTGGATATATCACCGATGGTAAAAAAGAT  300
             ||||||||||||||| |||||||||||||||||||||||||| || || ||||||||||||
Sbjct  241   TTGACTGGAAATGCGGCAATTGAGGAATTAACTGGATATATTACTGACGGTAAAAAAGAT  300

Query  301   GATGTTGAAGTTTTGCGTCGAGTGATGATGCGAGACCTTGAATGTGGTGCTTCAGTATCT  360
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Sbjct  301   GATGTTGAAGTTTTGCGTCGGGTGATGATGCGAGACCTTGAATGCGGTGCTTCAGTATCT  360

Query  361   ATTGCAAACAAAGTTTGGCCAGGTTTAATTCCTGAACAACCTCAAATGCTCGCAAGTTCT  420
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Sbjct  361   ATTGCAAACAAAGTTTGGCCAGGTTTAATTCCTGAACAACCTCAAATGCTTGCAAGTTCT  420

Query  421   TATGATGAAAAAGGCATTAATAAGAATATCAAATTTCCAGCCTTTGCTCAGTTAAAAGCT  480
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Sbjct  421   TATGATGAAAAAGGCATTAATAAGAATATCAAATTTCCAGCCTTTGCCCAGTTAAAAGCT  480

Query  481   GATGGAGCTCGGTGTTTTGCTGAAGTTAGAGGTGATGAATTAGATGATGTTCGTCTTTTA  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481   GATGGAGCTCGGTGTTTTGCTGAAGTTAGAGGTGATGAATTAGATGATGTTCGTCTTTTA  540
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Query  541   TCACGAGCTGGTAATGAATATCTAGGATTAGATCTTCTTAAGGAAGAGTTAATTAAAATG  600
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||
Sbjct  541   TCACGAGCTGGTAATGAATATCTAGGATTAGATCTTCTTAAGGAAGAGTTAATCAAAATG  600

Query  601   ACCGCTGAAGCCCGCCAGATTCATCCAGAAGGTGTGTTGATTGATGGCGAATTGGTATAC  660
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Query  661   CATGAGCAAGTTAAAAAGGAGCCAGAAGGCCTAGATTTTCTTTTTGATGCTTATCCTGAA  720
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Query  721   AACAGTAAAGCTAAAGAATTCGCCGAAGTAGCTGAATCACGTACTGCTTCTAATGGAATC  780
              |  |||||||||||||||||||||||||||||||||||||||||||| ||||||||| |||
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Query  781   GCCAATAAATCTTTAAAGGGAACCATTTCTGAAAAAGAAGCACAATGCATGAAGTTTCAG  840
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Query  901   GATGTACGTTTTTCTAAACTAGAACAAATGACATCTGGATATGATAAAGTAATTTTAATT  960
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Sbjct  901   GATGTACGTTTTTCTAAACTAGAACAAATGACATCAGGTTATGATAAAGTAATTTTAATT  960

Query  961   GAAAACCAGGTAGTAAATAACCTAGATGAAGCTAAGGTAATTTATAAAAAGTATATTGAC  1020
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Query  1021  CAAGGTCTTGAAGGTATTATTCTCAAAAATATCGATGGATTATGGGAAAATGCTCGTTCA  1080
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Query  1081  AAAAATCTTTATAAATTTAAAGAAGTAATTGATGTTGATTTAAAAATTGTAGGAATTTAT  1140
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Query  1141  CCTCACCGTAAAGACCCTACTAAAGCGGGTGGATTTATTCTTGAGTCAGAGTGTGGAAAA  1200
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Query  1201  ATTAAGGTAAATGCTGGTTCAGGCTTAAAAGATAAAGCCGGTGTAAAATCGCATGAACTT  1260
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Query  1261  GACCGTACTCGCATTATGGAAAACCAAAATTATTATATTGGAAAAATTCTAGAGTGCGAA  1320
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Query  1321  TGCAACGGTTGGTTAAAATCTGATGGCCGCACTGATTACGTTAAATTATTTCTTCCGATT  1380
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Query  1381  GCGATTCGTTTACGTGAAGATAAAACTAAAGCTAATACATTCGAAGATGTATTTGGTGAT  1440
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Sbjct  1381  GCGATTCGTTTACGTGAAGATAAAACTAAAGCTAATACATTCGAGGATGTATTTGGTGAT  1440

Query  1441  TTTCATGAGGTAACTGGTCTATGA  1464
              |||| ||||||||||||||||||
Sbjct  1441  TTTCGTGAGGTAACTGGTCTATGA  1464
```

Table 1. A comparison at nucleotide level between the 1st five matches of our nucleotide sequence

Accession no.	description	Max score	Query covarage	E.value	Identities	gaps
HM997020.1	wV7, complete genome	2543	100%	0.0	1435/1464 (98%)	0/1464 (0%)
HM035025.1	Shfl2, complete genome	2510	100%	0.0	1430/1465 (98%)	2/1465 (0%)
HM137666.1	T4T, complete genome	2510	100%	0.0	1429/1464 (98%)	0/1464 (0%)
AF158101.6	T4, complete genome	2510	100%	0.0	1429/1464 (98%)	0/1464 (0%)
X00039.1	T4 ligase gene	2510	100%	0.0	1429/1464 (98%)	0/1464 (0%)

Our nucleotide sequence was translated using expasy-translating tool, the right frame was blasted against protein database at NCBI, showing that the enzyme is a bacteriophage DNA ligase with some differences from all related published bacteriophage ligases as it is shown by the comparison between the 1st six matches of our protein sequence (table 2), these differences do not affect the functional domains for ligation activity.

Table 2. A comparison at protein level between the 1st six matches of our protein sequence.

Accession no.	Phage name	Max score	Query covarage	E.value	Identities	positives	gaps
AEK12454.1	ime09	986	100%	0.0	482/487 (99%)	484/487 (99%)	0/487 (0%)
YP_002854154.1	ime09	986	100%	0.0	482/487 (99%)	483/487 (99%)	0/487 (0%)
AEM00859.1	wV7	985	100%	0.0	481/487 (99%)	483/487 (99%)	0/487 (0%)
YP_004415092.1	Shfl2	984	100%	0.0	480/487 (99%)	484/487 (99%)	0/487 (0%)
YP_002854533.1	RB14	984	100%	0.0	480/487 (99%)	483/487 (99%)	0/487 (0%)
NP_049813.1	T4	984	100%	0.0	481/487 (99%)	483/487 (99%)	0/487 (0%)

Blast results in the table suggest that the SHPh it could be a new bacteriophage which has similarities to the published ones, and differs from them in locations that may have a role in evolutionary trends for this bacteriophage, without affecting the ligation activity of the enzyme.

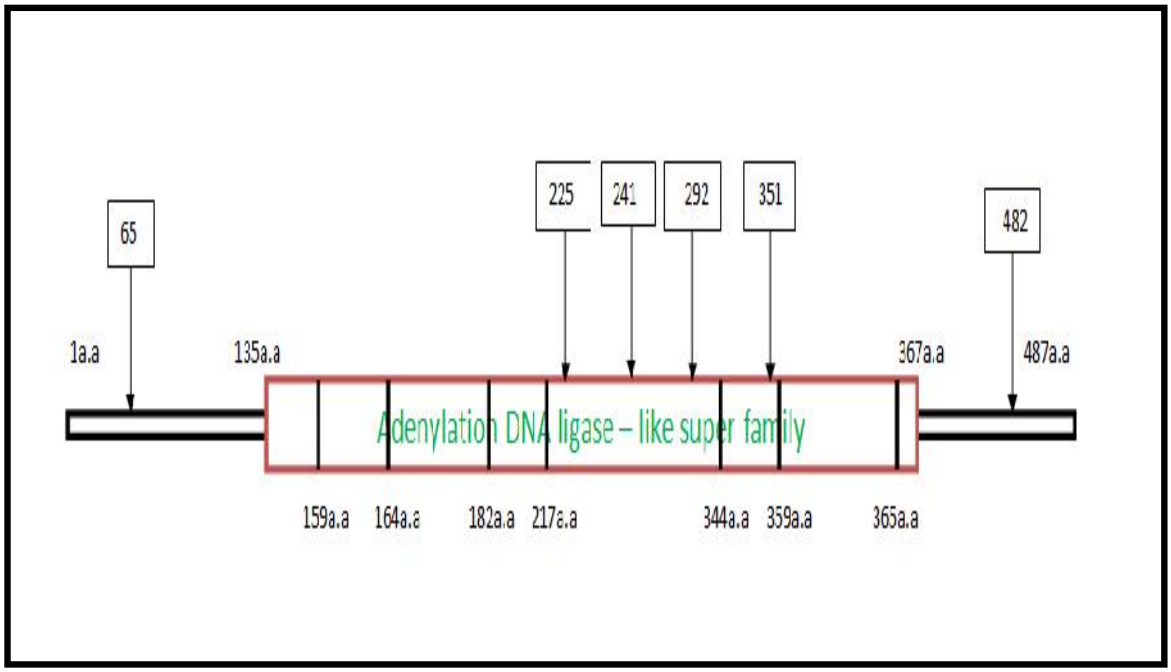

Fig.13 Sequence annotation of T4 DNA ligase Amino acid differences between our protein sequence and the published bacteriophage DNA ligase. The red block is a central domain that contains an adenylation DNA ligase- like superfamily, the black lines with the numbers 159, 164, 182, 217, 344, 359, and 365 are active sites in the domain, while the arrows represent a.a differences as following; a.a 65 (L/I), 225 (K/E), 241 (N/I), 292 (S/G),351 (I/T), and 482 (H/R).

4.6 The DNA ligase gene was expressed and its product was purified

The *DH5α* clones containing the expression vector was cultured and IPTG induced for ligase expression. The protein was purified using Nickel affinity column and protein fragments were visualized by SDS-PAGE gel electrophoresis. Bands matched the correct expected protein size of the DNA ligase and were about 55 KDa was produced and appeared in lanes loaded with eluted proteins (Figure 13).

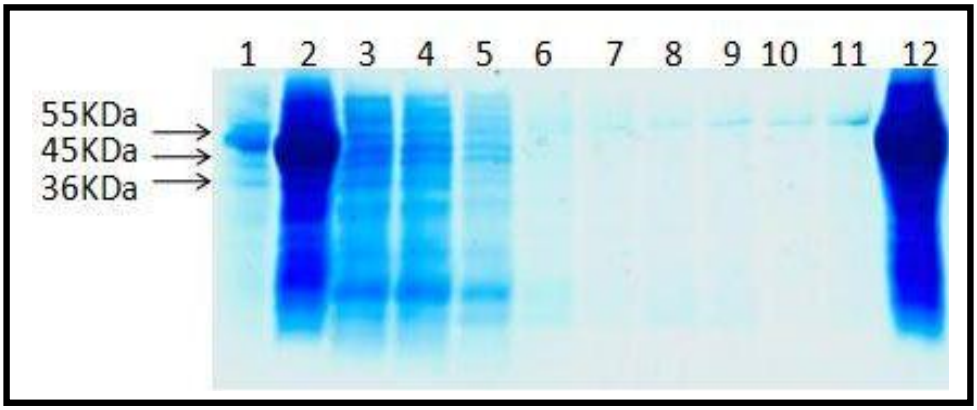

Figure 12. SDS -PAGE photo of the purirfied protein.

Visualization of the purified DNA ligase protein as appeared on 10% resolving SDS-PAGE gel. Lane 1 contains high molecular weight protein ladder (Sigma/ S8320). Lane 2 shows the purified protein from pellet. Lane 3 from flow through. Lane 4, 5, 6 from washing 1, washing 2, and washing 3 respectively. Lane 7, 8, 9, 10 from elution 1, elution 2, elution 3, and elution 4, respectively. Lane 11 from elution 2 from different purification. Lane 12 from pellet from different purification. protein band is appeared in the elution lanes that match the corresponding size in the protein ladder. The protein size is about 55.3 KDa.

CHAPTER FIVE

DISCUSSION

5.1 A strain (*DH5α*) specific bacteriophage was isolated

The starting material of this project obtained from a wastewater sample collected from Hebron Sewage System located in the south area of the city. Wastewater was used as a source for bacteriophage because it is a very rich natural environment of bacteria, particularly *E. coli*, and so on the presence of bacteria is an indicator of bacteriophage abundance because the phages exist where bacteria exist (Havelaar and Hogeboom, 1983).

An O\N culture of *DH5α* was infected with a wastewater sample. After multiple incubation and subculturing using new media, it is assumed that if a limited number of the phage is capable of infecting the *DH5α* strain, then after multiple cultures the number of these phages will multiply. The *DH5α* is an engineered commercial strain that is supposed to resist bacteriophages. The fact that the enriched phage particles grown on this strain means that a *DH5α* strain-specific bacteriophage has been isolated.

Plaque assays were performed using two types of bacteria, the commercial strain *DH5α* and the native *E.coli*. This phage is apparently capable of recognizing surface receptor proteins specific for *DH5α* strain.

The chloroform was added to facilitate the releasing of free bacteriophages form non-lysed infected cells by disrupting their cellular membranes. Centrifugation at high speed sediment all bacterial particles leaving the floating free bacteriophages (Havelaar and Hogeboom, 1983).

5.2 T4 DNA ligase gene was successfully cloned into *E.coli*

The genomic DNA of a bacteriophage was isolated using EZ- DNA kit. T4 DNA ligase gene with accession number X00039, of about 1.5 Kb was amplified using specific forward and reverse primers (T4 F a and T4 R a respectively), which were designed by using PerlPrimer v1.1.16 software. After band visualization by UV light on agarose gel; it was excised from the gel and purified by using Gel Purification Kit (Bioneer, K-3035) in order to be cloned in a cloning vector pGEM®-T-Easy, and in an expression vector pPROEX HTb.

pGEM®-T-Easy is a high copy number cloning vector, which has 3'-T overhangs at its insertion site (Promeaga, technical manual) that facilitates ligation and cloning of a PCR amplified insert that has complementary 3'-A overhang which is provided to both sides by *Taq* polymerase (Zhou and Gomez-Sanchez, 2000).

Then the recombinant vector was transformed by heat shock in *DH5α* competent cells which were prepared by ice- cold calcium chloride solution method (Tang et al., 1994). Calcium Chloride ($CaCl_2$) has the ability to create pores into bacterial cell membrane at allow entering of hydrophilic DNA molecule. Then the transformed cells were grown on a selective media containing ampicillin, X-gal and IPTG.

Cloning in pGEM® -T Easy vector depends on a blue- white selection, because it contains two selectable markers.

The 1st one is an ampicillin resistant gene which indicates the positive transformed cells, since only the transformed cells will grow on a media containing ampicillin.

The 2nd indicator gene is a *lac Z* which produces β- galactosidase activity (Song et al., 1994) that is responsible for breaking X-gal in the media producing blue color that is induced by availability of IPTG.

Lac Z gene is the place that the target gene will be inserted inside, so if it is inserted inside it, the *Lac Z* gene will be interrupted, thus the activity of β- galactosidase will not be observed. This will result in the appearance of white colonies, indicative of positive cloning. Otherwise it will produce blue colonies that indicate negative cloning since the target gene was not inserted inside *lac Z*, and thus the β-galactosidase activity is still on.

To allow affinity purification of the cloned DNA ligase, a subcloning into the pPROEX HTb, which is a homemade expression vector was carried out. This expression vector is a medium size of about 4779 bp with ampicillin resistant gene and N-terminus six tandem histidine (6xHis) residues) to facilitate purification on Nickel ion NTA (Ni-NTA)-containing columns (invitrogen, user manual, 2006).

The expression in pPROEX HTb will produce high level of desired recombinant protein with N- terminal 6- his tag, which will bind to the nickel ions in the column

and later displaced by a high concentration of imidazole and thus allowing the protein's purification (Hoffmann and Roeder, 1991).

In order to fix the expression direction in the right frame, the DNA insert was amplified by forward primer with *BamHI* restriction site (T4 F a), and reverse primer with *XhoI* restriction site (T4 R d), to be compatible with pPROEX HTb construction map as seen in figure 6. This cloning is directional and keeps the coding sequence in frame for correct expression of the recombinant protein.

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5.3 Sequence analysis

Our nucleotide sequence was aligned with X00039.1 cds (T4 DNA ligase) sequence that was published in the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/), nucleotide comparison showed some variations, most of them are in the 3rd letter in the codon, which means there is a small chance that the amino acid could be different.

The sequence was translated using expasy translation tool (http://www.expasy.ch/tools/dna.html), then the resulted protein sequence was blasted against protein database at NCBI using protein Basic Local Alignment Search Tool (BLAST) and it gives that SHPh is a bacteriophage ligase doesn't match 100% any one of a published bacteriophage ligases and this idea suggested that this ligase sequence belongs to a new bacteriophage which is somehow similar to the published ones, and differs from them in locations that may have a role in evolutionary trends for this bacteriophage, without affecting the ligation activity of the enzyme.

This step will open the revenues to discover a new bacteriophage that it could be colonize our area from ancient time and it is evolutionary different from what is published until now, this process it needs further work in order to identify what a bacteriophage kind.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

The gene of the study, which is a T4 DNA ligase is a very essential enzyme that has its own marks in the development of recombinant DNA technology.

Due to its role in modern DNA recombinant technology, there is a high demand on DNA ligase to allow the ligation of target DNA inserts into a chosen vector as part of DNA cloning technology, and despite it is commercially available from abroad, still it is difficult to secure a stable supply of it for researchers in Palestine, so we make the effort produce it from scratch.

The gene was isolated, cloned and expressed. The protein was purified but is still under quality control tests to fix ligation mechanism parameters.

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