Cloning and Expression of Phosphopentomutase and Cytidylate kinase from *Thermus thermophilus*

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In

Biotechnology

Submitted by:

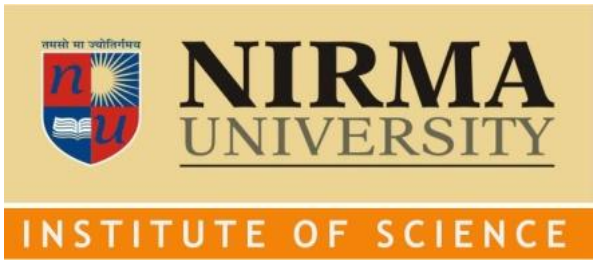
MANSI PARASRAMPURIA [12MBT013]

AND

DEEKSHA SINGHAL [12MBT014]

Under the guidance of

Dr. Mili Das



Institute of Science

Nirma University

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DECLARATION

We declare that the thesis entitled **"Cloning & Expression of Phosphopentomutase & Cytidylate kinase from *Thermus thermophilus*"** has been prepared by us, **Mansi Parasrampuria (12MBT013)** and **Deeksha Singhal (12MBT014)** under the guidance of **Dr. Mili Das**, Assistant Professor of Institute of Science, Nirma University, Ahmedabad. No part of this thesis has formed the basis for the award of any other degree or diploma.

Mansi Parasrampuria	**Deeksha Singhal**

Institute of Science,
Nirma University,
Sarkhej, Gandhinagar Highway,
Ahmedabad – 382481

Date:

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MANSI PARASRAMPURIA [12MBT013]

***DEEKSHA SINGHAL [12MBT014*]**

ABSTRACT

Extremophiles are unconventional microorganisms that can thrive in extreme environments. They are structurally adapted at the molecular level to withstand any harsh conditions. The biocatalysts, called extremozymes, produced by these microorganisms, are proteins that remain stable and function under extreme conditions. Due to their extreme stability, extremozymes offer new opportunities for biocatalysis and biotransformation, hence, are of great importance for industrial processes and scientific research. The extremophile that we selected is *Thermus thermophilus*, having an optimum growth temperature between 60°C. Bacterial phosphopentomutases (*deoB*) are alkaline phosphatase superfamily members that interconvert α-D-ribose-5-phosphate (ribose-5-phosphate) and α-D-ribose-1-phosphate (ribose-1-phosphate). Phosphopentomutase is a key enzyme in nucleoside catabolism. Cytidylate kinase enzyme belongs to the family of phosphotransferases. This enzyme participates in pyrimidine metabolism. Among mesophilic organisms like *Streptococcus mutants*, cytidylate kinase has been found to be an excellent antibiotic target.

Our objective was to clone and express, selected extremophilic proteins into an expression vector. The phosphopentomutase and cytidylate kinase genes were selectively amplified. TA cloning method was selected. The amplified genes were first cloned into TA cloning (pTZ57R/T) vector and further they were subcloned into the expression vector, pET28a^{+}. For this, vector and insert DNA were double digested with restriction enzymes and ligated with the help of T4 DNA ligase enzyme. *E.coli* BL21 (DE3) was used for the expression of the clones. But ligation of insert and vector was not successfully done.

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1. INTRODUCTION

1.1 PROTEINS

native state Proteins are molecular devices, in the nanometer scale, where biological function is exerted (Breda *et al.*, 2008). More than 100,000 proteins in our bodies are produced from a set of only 20 building blocks, known as amino acids (Berg *et al*., 2002).

Biologically active proteins are the polymers consisting of amino acids linked by covalent peptide bonds and are complex in nature that is defined in four levels of structure (Campbell and Farrell, 2008). All structural and functional properties of proteins are derived from the chemical properties of the polypeptide chain (Carey and Hanley, www.biophysics.org). Some proteins are quite rigid, whereas others display a considerable flexibility. Proteins with some flexibility may act as hinges, springs, or levers that are crucial to protein function, to the assembly of proteins with one another and with other molecules into complex units, and to the transmission of information within and between cells (Berg *et al.*, 2002). How does a protein find itswithout a globally exhaustive search (Dill *et al.*, 1993)?

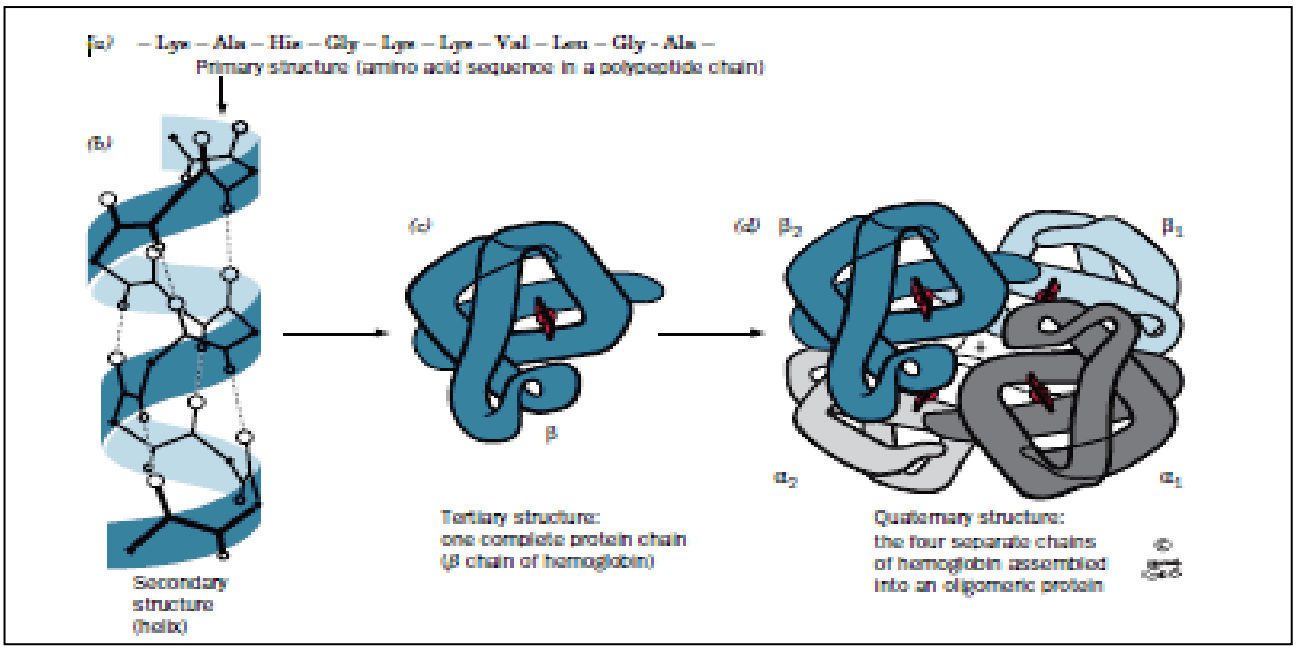

FIGURE 1: PROTEIN STRUCTURE

(a) Primary structure (b) Secondary structure (c) tertiary structure (d) quaternary structure

1.1.1 PROTEIN-FOLDING MECHANISM

Proteins spontaneously fold up into three-dimensional structures that are determined by the sequence of amino acids in the protein polymer. Protein function is directly dependent on this three-dimensional structure. Thus, proteins are the embodiment of the transition from the one-dimensional world of sequences to the three- dimensional world of molecules capable of diverse activities (Stryer *et al.*, 2002). The classic principle of protein folding is that all the information required for a protein to adopt the correct three-dimensional conformation is provided by its amino acid sequence (Cooper and Sunderland, 2000). Thus transition of protein molecule to its native state from a denatured state is called protein folding (Behe *et al*., 1991).

Native fold is stabilized by chemical forces both within the protein and between the protein and its surrounding environment. Thus native fold of each protein has evolved to be stable because they are highly complex and irregular. Proteins larger than 100 residues in length fold on a much rougher energy surface in which folding intermediates are commonly populated en route to the native state. The reason for this seems to be that larger chains have a higher tendency to collapse in aqueous solvent, resulting in the formation of compact states that may contain substantial elements of native-like structure (Jahn and Radford, 2005). Kinetic stability plays a significant role in determining the level of functional protein because it reflects the energy levels of folding intermediates between a protein's folded native state, unfolded states and/or misfolded states, which in turn can significantly control the rate of a protein's folding/unfolding, aggregation and degradation (Socha and Tokuriki, 2013).

1.1.2 FACTORS CONTRIBUTING TO THE STABILITY OF PROTEINS

Thermostability of a protein is a property which cannot be attributed to the presence of a particular amino acid or to a post synthetic modification. Some of the proposed mechanisms/indicators of increased thermostability include : a more highly

hydrophobic core ,tighter packing or compactness, deleted or shortened loops, greater rigidity(for example through increased Proline content in loops), higher secondary structure content, greater polar surface area, fewer and/or smaller voids, smaller surface area to volume ratio, fewer thermolabile residues, increased hydrogen bonding, higher isoelectric point & more salt bridges/ion pairs and network of salt bridges (Taylor & Vaisman, 2009).

1.2 EXTREMOPHILES

Extremophiles are organisms that are adapted to grow optimally at or near to the extreme ranges of environmental variables. Most extremophiles are microorganism that thrive under conditions that, from a human perspective, are clearly hostile (Horikoshi and Bull, 2011). RD MacElory first coined the term extremophile in 1974, to designate any organism that are able to support environmental conditions usually fatal to most eukaryotic cells (Berlemont and Gerday, 2011). A much larger diversity of organism are known that can tolerate extreme conditions and grow, but not optimally in extreme habitats; these organisms are defined as extremotrophs (Muller *et al*., 2005).

An extremophile is an organism that thrives under extreme conditions, which is frequently used to refer to organisms that are unicellular and prokaryotic (Hough and Danson, 1999). All extremophiles are not unicellular.

Now extremophilic organism can be classified depending on their optimal growth conditions, i.e. thermophiles, psychrophiles, acidophiles, alkalophiles, halophiles and barophiles, respectively (Stetter, 1999). Thermophilic and hyperthermophilic organisms live and survive at high (up to 75°C) and very high temperatures (up to 115°C), respectively (Kumar and Nussinov, 2001). Psychrophile term was first coined in 1902, have been found inhabiting temperatures below 0°C. The novel molecules first isolated from psychrophilic or psychrotolerant microorganisms are secondary metabolites, which are largely based on modified peptides (Life at the extremes, 2008). Acidophiles and alkalophiles live and survive at

extreme acidic or basic values of pH (Madigan and Marrs, 1997). Halophilic organisms, live in the presence of high salt concentrations (5–30%) (Madigan and Marrs, 1997; Rothschild and Manicinelli, 2001). Some extremophilic organisms are also able to live in the presence of high metal ion concentrations (metallophiles), or high radiation levels (radiophiles), or in the absence of oxygen (Champdore *et al*., 2006). Barophiles also called as piezophiles are organisms which thrive at high pressures such as deep sea bacteria or archaea. They are generally found on ocean floors, where pressure often exceeds 380 atm (Carolina *et al*., 1999).

1.3 EXTREMOZYMES

Extremophiles are a source of enzymes called extremozymes with extreme stability and biocatalysts activity which are attractive because they are stable and active under conditions that were previously regarded as incompatible with biological materials (Hough and Danson, 1999). These extremozymes are expected to fill the gap between biological and chemical processes due to their unusual properties (Fujiwara S, 2002).

Many attempts have been made to understand the stability of extremozymes in terms of their three-dimensional (3D) structure. This approach requires high-resolution structural data for homologous enzymes from both mesophiles and extremophiles so that differences, which might result in enhanced stability of the extremozyme, can be identified by structural comparision (Hough and Danson, 1999). Mesophilic enzymes are often not well suited for the harsh reaction conditions required in industrial processes because of the lack of enzyme stability (Demirjian *et al.*, 2001).

1.4 THERMOPHILES

Themophiles, a subgroup of extremophiles, are structurally adapted at the molecular level to withstand extreme heat (Rothschild and Manicinelli, 2001) The discovery of *Thermus aquaticus* in the hot springs of Yellowstone in the 1960‟s led towards the study of organisms that thrive at very high temperatures (Taylor *et al*., 2010). Organism with T_{max} > 50°C have been called thermophilic. Brock had

suggested a definition of a thermophilic boundry at 55 to 60°C. Thermophilic range is further divided from 55-60°C to 80-85°C and hyperthermophiles as those which can grow optimally above 80-85°C (Kristjansson and Stetter, 1992)

1.4.1 Resistance of thermophiles to high temperatures and denaturation

- Thermophiles are reported to contain proteins which are thermostable and resist denaturation and proteolysis (Kumar and Nussinov, 2001).
- Specialized proteins known as chaperonins are produced by these organisms, which help, after their denaturation to refold the proteins to their native form and restore their functions (Everly and Alberto, 2000).
- Proteins of thermophiles have increased surface charge and less exposed thermolabile amino acids. Thus, increased ionic interaction and hydrogen bonds, increased hydrophobicity, decreased flexibility and smaller surface loops confer stability on the thermophilic protein (Joseph Gomes *et al., 2004).*
- The cell membrane of thermophiles is made up of saturated fatty acids. The fatty acid provides a hydrophobic environment for the cell and keeps the cell rigid enough to live at elevated temperatures (Herbert and Sharp, 1992).
- The DNA of thermophiles contains a reverse DNA gyrase which produces positive super coils in the DNA (Lopez, 1999). This raises the melting point of the DNA (the temperature at which the strands of the double helix separate) to at least as high as the organisms maximum temperature for growth.

1.4.2 THERMOZYMES

Proteins produced by thermo and hyperthermophilic microorganisms, growing between 45 and 110°C and are more resistant to thermal and chemical denaturation. Thermophilic proteins are more resistant to proteolysis and chemical denaturation; they denature at a much higher temperature than regular mesophilic proteins (Engel *et al*., 1998; Eijsink *et al*., 1998; Van den Berg, 2003). Thus thermostability of enzymes

from thermophiles can be as high as up to 140°C (Adams and Kelly, 1995). Proteins are individuals that accumulate increments of stabilization; in thermophiles these come from charge clusters, networks of hydrogen bonds, optimization of packing and hydrophobic interactions, each in its own way (Jaenicke and Böhm, 1998). Most protein-stabilization mechanisms (i.e. hydrophobic interactions, packing efficiency, salt-bridges, hydrogen bonds, reduction of conformational strain, reduction of the entropy of unfolding, α-helix stabilization, loop stabilization and resistance to covalent destruction) have been identified by stability studies using mesophilic models. Recent structural comparisons between mesophilic enzymes and thermozymes validate these mechanisms (Vieille and Zeikus, 1996).

The observed structural resistance may reflect a restriction on the flexibility of these proteins, which, allows them to be functionally competent at elevated temperatures, renders them unsually rigid at mesophilic temperatures (10–45°C).

In thermophilic proteins a number of amino acids are often exchanged. These exchanges with some strategic placement of proline in β-turns give rise to a stabilization of the protein. Mutagenesis experiments have confirmed this statement. Comparative analysis of the X-ray structures available for several families of proteins, including at least one thermophilic structure in each case, it has appeared that thermal stabilization is accompanied by an increase in hydrogen bonds and salt bridges (Scandurra *et al.,* 1998). The proteins appeared to be somewhat shorter than those in mesophiles (Das *et al*., 2000).

1.5 *Thermus thermophilus*

Thermus thermophilus is an extremely thermophilic, halotolerant bacterium. It was originally isolated from a natural thermal environment in Japan (Oshima and Imahori, 1974). The genome of this organism consists of a 1,894,877 base pair chromosome and a 232,605 base pair mega plasmid, which is designated as pTT27. It contains 2127482 base pair where 1476627 base pairs (69.40%) are G+C content which allows the bacterium to strive in extreme thermo environment. In addition, this bacterium consist total number of 2210 protein encoding genes and 53 RNA genes

(Lioliou *et al*., 2004). *T. thermophilus* uses various proteinaceous substrates as well as carbohydrates for growth. These are made available by numerous (exo) proteases, lipases, pullulanases, α- and β-glucosidases and galactosidases, genes of which were identified in the genome sequence (Henne *et al*., 2004**)**. The 2,218 identified putative genes from *T. thermophilus* were compared to those of the closest relative sequenced so far, the mesophilic bacterium *Deinococcus radiodurans* (Lioliou *et al*., 2004).

The cells of *Thermus thermophilus* are gram-negative, nonsporulating and aerobic rods containing yellow pigment. The optimum temperature for growth is between 65 and 72°C, the maximum being 85°C and the minimum being 47°C (Brock and Freeze, 1969) This microorganism is sensitive to various antibiotics, even to those which are known to be rather ineffective against gram-negative bacteria. When the intact cells are treated with egg-white lysozyme at 60°C Spheroplast-like bodies are formed (Oshima and Imahori, 1974).

1.6 ENZYMES UNDER STUDY

1.6.1 PHOSPHOPENTOMUTASE

Enzyme-catalyzed phosphoryl transfer forms the basis for many biological, bioenergetic and regulatory processes and is one of the most common cellular reactions (Knowles, 1980). Numerous enzyme families have evolved mechanistically distinct solutions for phosphoryl transfer (Allen and Mariano, 2004). Bacterial phosphopentomutases (EC 5.4.2.7) interconvert ribose-1-phosphate and ribose-5-phosphate, which bridges glucose metabolism and RNA biosynthesis. Phosphopentomutase is an enzyme that catalyzes the following chemical reaction

α-D-ribose-1-phosphate → D-ribose-5-phosphate

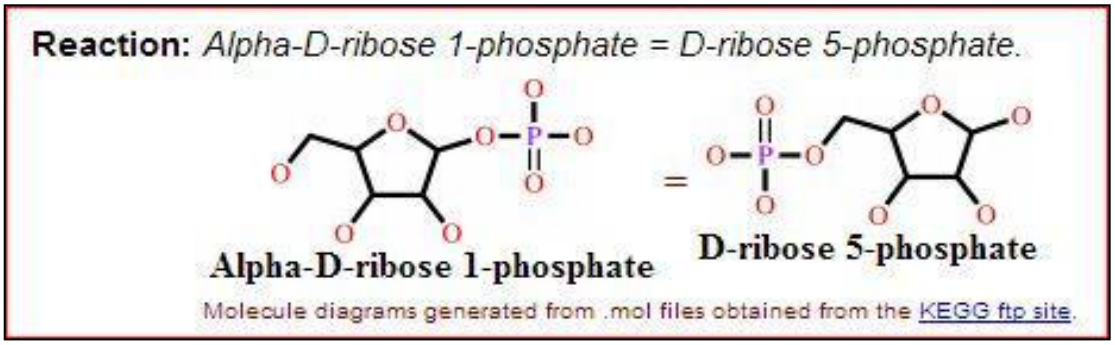

FIGURE 1.6.1: KEGG database pathway for phosphopentomutase. Here *deoB* gene codes for phosphopentomutase.

Phosphomutases are phosphotransfer enzymes that rearrange the position of phosphate within substrate molecule through either intramolecular (*i.e.* the phosphate is transferred to a different position on the same molecule) or intermolecular phosphoryl transfer (*i.e.* the phosphate is transferred from one substrate molecule to another). Phosphopentomutase appear to be biochemically and structurally distinct from their human congeners (Hammer and Peterson, 1970; Galperin *et al.*, 1998) making them potential targets for antibiotic development.

1.6.2 CYTIDYLATE KINASE

Pyrimidines are intimately involved in the physiology of cells. They participate at multiple levels in intermediary and secondary metabolism from nucleotide and macromolecule biosynthesis to the biosynthesis of complex carbohydrates and the metabolic regulation of intermediary metabolism. All pyrimidines within the cell are derived from UMP, which arises either from the de novo pyrimidine biosynthetic pathway or from salvage of preformed pyrimidines. UMP/CMP kinase converts uridine and cytidine monophosphates into the corresponding uridine and cytidine diphosphates. Because all pyrimidines are derived from UMP, UMP kinase is the first committed step and one of the central enzymes in the further anabolism of pyrimidine nucleotides.

Cytidylate kinase (EC 2.7.4.14) is an enzyme that catalyses the phosphoryl transfer from ATP to CMP and dCMP, resulting in the formation of nucleoside diphosphates.

$$ATP + (d)\,CMP \leftrightarrow ADP + (d)\,CDP$$

Thus, the two substrates of this enzyme are ATP and dCMP, while its two products are ADP and dCDP. In eukaryotes, CMP/UMP kinase catalyses the conversion of UMP and CMP to UDP and CDP, respectively, with high efficiency. This enzyme participates in pyrimidine metabolism (Bucurenci *et al*., 1995). CMP kinase plays a key role in cellular nucleic acid synthesis. Several crystal structures of CMP/UMP kinases have been determined including CMP kinase from *Escherichia coli* (Briozzo *et al*., 1998; Bertrand *et al*., 2002), UMP/CMP kinase from *Dictyosteliumdiscoideum* (Scheffzek *et al*., 1996; Schlichting and Reinstein, 1997), and UMP kinase from *Saccharomyces cerevisiae* (Müller-Dieckmann and Schulz, 1994). This *S. Pneumoniae* CMP kinase thus represents an attractive drug target for developing novel antibiotics to overcome the problems of drug resistance for this organism.

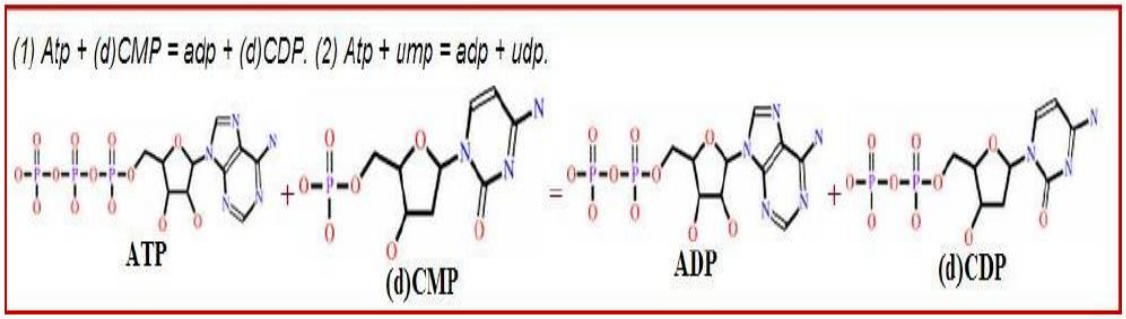

FIGURE 1.6.2: KEGG database pathway for cytidylate kinase. *cmk* gene codes for cytidylate kinase
REFERENCE : KEGG database site

Thermophilic enzymes are potentially applicable in a wide range of industrial processes mainly due to their extraordinary operational stability at high temperatures and denaturant tolerance. Such enzymes are used in the chemical, food, pharmaceutical, paper, textile and other industries (Pernilla Turner *et al.*, 2007). More than 3000 different enzymes have been identified. Despite their many advantages, the applications of these enzymes are often restricted because of their limited stability to the harsh environments typically used in industrial processes. Therefore, the discovery of organisms adapted to such extreme conditions offer new opportunities for biocatalysis and biotransformation, sparking great interest in their extremely stable enzymes.

Each different class of extremophile has unique features that can be exploited to provide biomolecules for the industry. High thermostability also promises to have greater tolerance to organic solvents and a longer useful life. The possibility of recovering volatile products directly from a culture provides the opportunity to develop simplified, elegant bioprocesses. However, a series of engineering problems remain to be solved (Sonnleitner and Fiechter, 1983).

Such enzymes can also be used as models for the understanding of thermostability and thermo-activity, which is useful for protein engineering (Haki and Rakshit, 2003)

2. Objectives

1) Cloning, Expression and purification of recombinants proteins.

2) Thermal stability of phosphopentomutase and cytidylate kinase.

3. MATERIALS

3.1 MATERIALS

The lyophilized cultures of the organism, *Thermus thermophilus* (MTCC-1496) was purchased from MTCC (Microbial Type Culture Collection). Plasmid DNA Mini kit, Minelute Gel Extraction Kit were obtained from Qiagen. Designed primers were procured from Sigma Alridge. Restriction enzymes (NdeI and XbaI), T4 DNA ligase, 1 kb DNA ladder, TA cloning kit and PCR grades i.e. Taq polymerase enzyme and dNTPs were obtained from Thermoscientific. RNase A was procured from Merck Biosciences. Ethidium Bromide, Agarose (Low EEO and Low melting), Luria Bertani Broth and Luria Bertani Agar were obtained from HiMedia. Plastic wares used were obtained from Tarsons. Expression vector pET28a^{+}, *Escherichia coli* DH5α and BL21 (DE3) were a kind gift received from Prof. Raghavan Varadarajan (Molecular Biophysics Unit, Indian Institute of Science, Bangalore, India).

3.1. Plasmid Maps

We used two plasmids (the TA vector-pTZ57R/T and the expression vector-pET28a^{+}) for our cloning purposes. Their plasmid maps are as follows:

3.1.1 pTZ57R/T vector

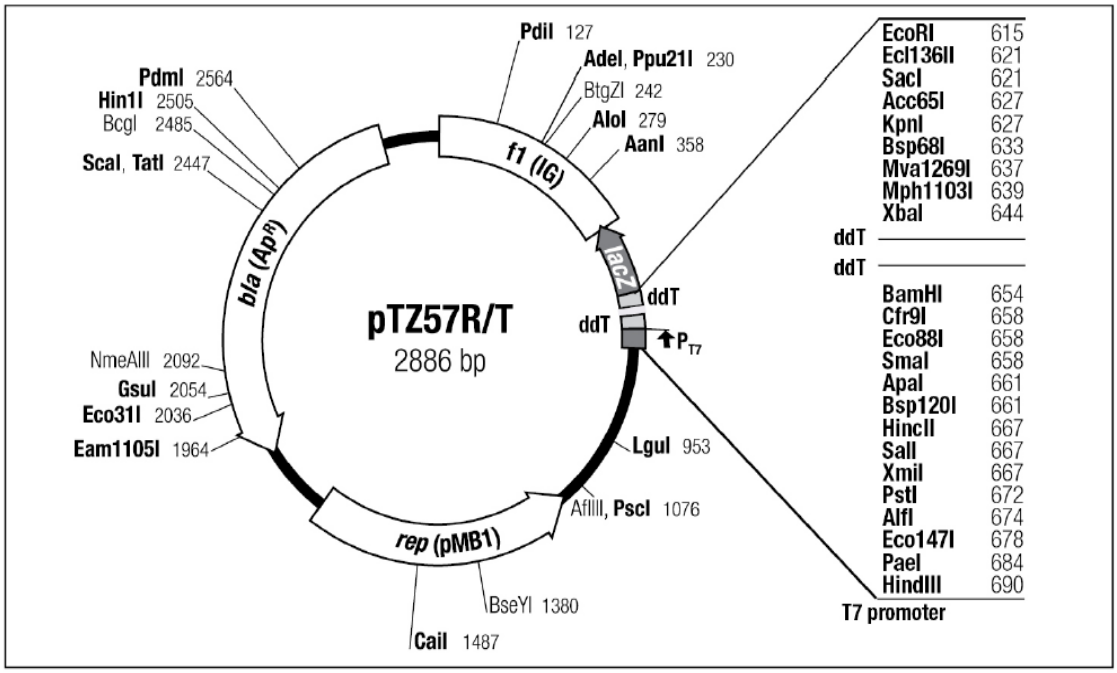

FIGURE 3.1.1 : pTZ57R/T Cloning Vector

Source: Thermo Scientific InsTAclone PCR Cloning Kit

Here pTZ57R/T vector have dT overhangs. PCR products have 3'-dA overhangs. It has terminal transferase activity of Taq DNA polymerase and other non-proofreading thermostable DNA polymerases. Such enzymes add a single 3'-A overhang to both ends of the PCR product. The structure of these PCR products favors direct cloning into a linearized cloning vector i.e. pTZ57R/T with single 3'-ddT overhangs.

3.1.2 pET28a^{+} vector

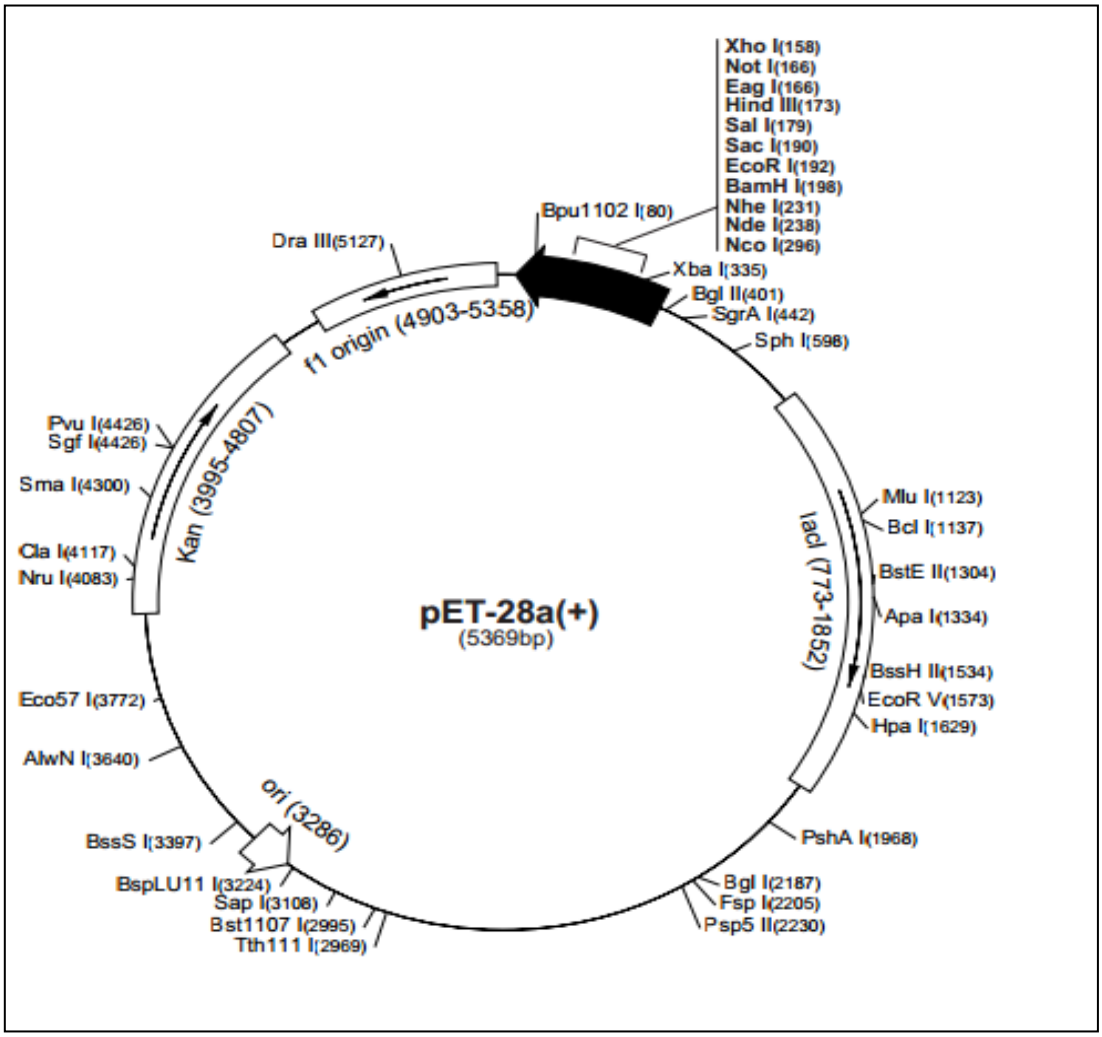

FIGURE 3.1.2 : pET-28a^{+} cloning/expression vector

Source : Novagen

deoB/cmk genes were inserted into expression vector by restricting both pET28a^{+} and pTZ57R/T harbouring *deoB/cmk* gene with restriction enzymes XbaI and NdeI having restriction sites which are present in multiple cloning site. The sites of restriction enzymes are:

- XbaI-TCTAGA
- NdeI-CCATAT

3.2 Gene sequence of the enzymes (source: NCBI)

The gene sequences were retrived from NCBI. Also *deoB* gene and *cmk* gene codes for phosphopentomutase and cytidylate kinase respectively which is also retrived from NCBI.

3.2.1 Phosphopentomutase

>gi|46198308:1572620-1573765 Thermus thermophilus HB27, complete genome

TTGAAGGCGGTGGCCATCGTTTTGGACTCCGTGGGCCTGGGCTACCTGCCC
GACGCCCCCCTCTTCGGCGACGAGGGGGCGGACACCTTGGACCACACGGT
CCTGAAGACCGGGATCGCCCTCCCCCACCTGGCCGGCCTCGGCCTCGGCC
GGGTCCCCGGGGTCCACACCCTGCCCCGCGCCGAGCGGCCCCGGGGCGGG
TTCGGCCGCATGCGGGAGGTGAACCCCGGCAAGGACACCACCACGGGGC
ACTGGGAGTTCGTGGGGATCCACCTGGAAAAGCCGTTCCGCACCTTTCCC
CAGGGGTTTCCCGAGGACGTTCTCCGGGAGTGGGCCGAGGCCATCGGGGT
GGGAGGGTGGCTTTTGAACCGCCCCTACTCGGGGACGGAGGCCATCCGCG
ACTACGGGGAGGCCCACCTCAAGACGGGCTACCCCATCGTCTACACCTCC
GCCGACAGCGTCTTCCAGGTGGCGGCCCACGTGGACGTGGTCCCCGTGGA
GGAGCTTTACCGCTTTTGCCAGGTGGCCCGGGAGAGGCTCGTGGGCGAGC
TCCAGGTGGCCCGGGTCATCGCCCGGCCCTTCGCCGGGGAGCCGGGGAGG
TTCTACCGGCTGGAGCACCTCCGGAAGGACTTCGCCCTCGAGCCCCCGAG
GAACGTCCTGGACGTCTTGCGGGAAGGAGGCCTCGAGGTGGTGGGGGTG
GGGAAGATCCCCGACATCTACGCGGGGCGCGGCTTCACTCGGAAGGTCAA
GACCAAGGACAACCAAGACGGCCTGGAGAAGACCCTGGCCCTCATGGGG
GAGCCTTTTTCCGGCCTCGTCTTCACCAACCTGGTGGACTTTGACTCCAAG
TACGGCCACCGCCGCGACCCCGAGGGGTACGGGAGGGCCCTCGTGGAGCT
GGACGCCTTCCTCCCGAGGCTTCTTGCCGCCTTGGGGCCCGAGGACCACCT
CTTCCTGGTCTCGGACCACGGCAACGACCCCACCTTCTTCGGCACCGACCA
CACCCGGGAGTACGCGATGCTCCTCTGGGTGGGGCCGGGGGTGGAGGGG
GAGCTGGGCACCCGGGAGACCTTCGCCGACCTCGGGGCCACCTGGGCCCG
CCTCTTCGGCCTGGCCTGGGACGGCCCCGGAACGAGCCTCGTCTGA

3.2.2 Cytidylate kinase

>gi|46198308:79387-80013 Thermus thermophilus HB27, complete genome

ATGCGCGGCATCGTGACCATAGACGGGCCTTCGGCCTCCGGCAAGAGCTC
CGTGGCCAGGCGGGTGGCGGCGGCCTTGGGCGTGCCCTACCTCTCCAGCG
GCCTCCTCTACCGGGCGGCCGCCTTCCTCGCCCTGAGGGCGGGGGTGGAC
CCCGGGGACGAGGAGGGGCTCCTCGCCCTCCTCGAGGGCCTGGGGGTGCG
CCTTTTGGCCCAGGCGGAGGGCAACCGGGTCCTGGCGGACGGGGAGGAC
CTCACCCCCTTCCTGCACACCCCCGAGGTGGACCGCGTGGTCTCGGCGGT
GGCCCGCCTCCCGGGGGTCCGGGCCTGGGTCAACCGCAGGCTCAAGGAGG
TGCCTCCGCCCTTCGTGGCCGAGGGGAGGGACATGGGCACGGCCGTCTTC
CCCGAGGCGGCCCACAAGTTCTACCTCACGGCGAGCCCCGAGGTGCGGGC
GTGGCGGCGCGCCCGGGAAAGGCCCCAGGCCTACGAGGAGGTGCTCCGG
GACCTCCTCCGGAGGGACGAGCGGGACAAGGCGCAAAGCGCCCCCGCTC
CCGACGCCCTCGTCCTGGACACCGGGGGGATGACCCTGGACGAGGTGGTG
GCCTGGGTCCTGGCCCACATCCGGAGGTAG

3.3 Primers

Primer sequences, to amplify the respective genes i.e. phosphopentomutase and cytidylate kinase from total genomic DNA of *Thermus thermophilus* were designed manually from the gene sequence of the enzymes and restriction sites were added to the 5‟ end:

3.3.1 Phosphopentomutase

Forward Primer – 5‟ TCTAGATTGAAGGCGGTGGGCCATCGTT 3‟

Reverse primer – 5‟ CATATGTCAGACGAGGCTCGTTCCGG 3‟

3.3.2 Cytidylate kinase

Forward primer – 5‟ TCTAGAATGCGCGGCATCGTGACCATA 3‟

Reverse primer – 5‟ CATATGCTACCTCCGGATGTGGGCCA 3‟

4. METHODS

4.1 TBP Broth for growth of *Thermus thermophilus* (MTCC Catalogue – 1496)

Composition	Amount(g)
Beef extract	0.4g
Polypeptone	0.4g
K_2HPO_4	0.3g
$KH_2 PO_4$	0.1g
Distilled water	100ml
pH	7.0

Revival of culture

The vial containing lyophilized culture of *Thermus thermophilus* was opened under aseptic condition and suspended in 0.5 ml of media i.e. TBP broth.The culture was inoculated in 100 ml medium and incubated at 60°C for 2-3 days. When OD of the culture reached 0.7-0.8, one loopful of culture was streaked on the agar plate i.e. TBP media (containing medium prescribed by MTCC-1496) by four flame method and incubated at 60°C. Moisture was maintained in the incubator by adding water to replace the loss during the incubation period (Tairo and Kazutomo, 1974)

Glycerol stocks were made to preserve our bacterial cells. For the glycerol stock preservation, in an autoclaved cryovial 150 µl of sterile glycerol was taken. To this vial 850 µl of culture of *Thermus thermophilus* (OD-0.8 at 600nm) was added under sterile condition. This vial was inverted several times for proper mixing. After covering with parafilm, the cryovial was stored at -20°C (Ausubel, 2002).

4.2 Principle of TA cloning

To proceed with indirect cloning, genes of interest were amplified from the genomic DNA of *thermus thermophilus* using manually designed primers having the restriction sites included in them. The amplified gene was cloned into pTZ57R/T (TA vector) vector.

- TA cloning is a system for direct one-step cloning of PCR products with 3'-dA overhangs. The high quality TA cloning vector pTZ57R/T is ready to use for efficient ligation with PCR products providing high cloning yields and low background. It has terminal transferase activity of Taq DNA polymerase and other non-proofreading thermostable DNA polymerases. Such enzymes add a single 3'-A overhang to both ends of the PCR product. The structure of these PCR products favors direct cloning into a linearized cloning vector with single 3'-ddT overhangs. Such overhangs at the vector cloning site not only facilitate cloning, but also prevent the recircularization of the vector. As a result, more than 90% of recombinant clones contain the vector with an insert. (Thermo Scientific InsTAclone PCR Cloning Kit).

The TA clones (pTZR57R/T harbouring *deoB/cmk*) and the expression vector pET28a^{+} were then double digested with the restriction enzymes NdeI and XbaI. The digested gene and the digested vector were then ligated.

PCR was set up for amplification of the genes of interest. For TA cloning, fresh PCR products with intact dA overhangs are necessary (Bing-Yuan Chen and James, 2002).

4.3 Genomic DNA isolation from *Thermus thermophilus*

- 50 ml of TBP broth was prepared for the given organism (*Thermus thermophilus)* and autoclaved. The media was inoculated with the revived culture and the cells were allowed to grow for 2 days at 60°C.
- 24 ml of the bacterial culture was taken in a microcentrifuge tube and was centrifuged at 7500 rpm for 2 minutes.
- Supernatant was discarded and the pellet was resuspended in 9.072 ml TE buffer. Also 480 µl of 10% SDS was mixed and the tubes were incubated at 37°C for an hour.
- 1.6 ml of 5 M NaCl was added and mixed. To this mixture, 1.28 ml of CTAB/NaCl was added and was mixed thoroughly by inverting it slowly. It was incubated for 10 minutes at 65°C in a waterbath.
- 1 volume (0.7-0.8 ml) of 24:1 chloroform/isoamyl alcohol was added and mixed thoroughly by inverting it slowly. Centrifugation of 4-5 minutes at 7500 rpm was done.
- Supernatant was transferred to a fresh tube and equal volume of 25:24:1 Phenol/Chloroform/Isoamyl alcohol was added and then mixed thoroughly by inverting it slowly and was microcentrifuged for 5 minutes at 7500 rpm.
- Supernatant was transferred to a fresh vial. To this 0.6 volume of isopropanol was added and mixed gently until a stringy white DNA precipitate was formed.
- Incubated on ice for 1 hr. Microcentrifugation was done for 20 minutes at 7500 rpm, 4°C.
- Supernatant was discarded and to the pellet 70% ethanol was added. Microcentrifugation was done for 20 minutes at 7500 rpm, 4°C.
- Pellet was allowed to dry. The dried pellet was resuspended in 50µl of TE buffer.

(Short Protocols in Molecular Biology, Volume 1).

The isolated DNA was made RNAse free by treating with RNase A (Ausubel, 2002). Then, concentration of the isolated genomic DNA was calculated by measuring absorbance at 260nm.

4.4 RNase treatment

RNase solution (0.572 µl) was first made DNase free by keeping the solution in boiling water bath for 15-20 minutes. Then to 143 µl of genomic DNA, 0.572 µl of RNase was added (40 µg/ml final concentration). It was incubated at 37°C for 30 minutes with occasional shaking. Then equal volume of Phenol/Chloroform/Isoamyl Alcohol (25:24:1) was added and centrifuged at 14,000 rpm for 5 minutes. Supernatant was transferred to a fresh tube and twice the volume of 99.5% absolute alcohol was added to precipitate DNA through centrifugation at 12,000 rpm for 2 minutes. Supernatant was discarded and the pellet was allowed to dry. To the dried pellet, 20 µl of TE buffer was added (Short Protocols in Molecular Biology, Volume 1, 1989).

4.5 Absorbance of DNA at 260 nm

To measure the concentration of DNA, appropriate dilutions were made with TE buffer (either 1:50 or 1:100). Spectrophotometer was standardize using TE buffer as blank. Absorbance of the sample was measured at 260 nm. The concentration of DNA in the sample was then calculated.

Concentration of DNA (µg/ml) = OD260 X 50 X dilution factor.

(Barbas III *et al*., 2001)

4.6 Amplification of the phosphopentomutase and cytidylate kinase genes

Polymerase Chain reaction was set up to amplify the desired genes from the genomic DNA which is isolated from *Thermus thermophilus*.

4.6.1 PCR amplification of phosphopentomutase

PCR REACTION MIXTURE	Volume
10x amplification buffer	2.5 µl
20 mM solution of four dNTPs (pH 8.0)	1 µl
20 µM forward primer	1 µl
Template DNA	1 µl (1 ng)
Taq polymerase	1.0 µl(1-2units)
Sterile MiliQ water	17.5 µl
Total volume	25 µl

PCR was carried out according to the following cycle

PCR Cycles	Thermal conditions
Initial denaturation	94°C, 5 minutes
Final denaturation	94°C, 1 minute
Annealing	65°C, 1 minute
Extension	72°C, 1.5 minutes
Final extension	72°C, 10 minutes
Number of cycles	30

(Sambrook and Russel, Molecular Cloning, 2001)

4.6.2 To amplify the gene of cytidylate kinase Polymerase Chain Reaction was set up as follows (Gradient PCR was set up)

PCR REACTION MIXTURE	VOLUME
10x amplification buffer	2.5 µl
20 mM solution of four dNTPs (pH 8.0)	1 µl
20 µM forward primer	1 µl
Template DNA	0.5 µl ,1 µl (1 ng)
Taq polymerase	1.0 µl(1-2units)
Sterile MiliQ water	17.5 µl, 18 µl
Total volume	25 µl

PCR was carried out according to the following cycle

PCR Cycles	Thermal conditions
Initial denaturation	94°C, 5 minutes
Final denaturation	94°C, 1 minute
Annealing	55°C, 60°C ,63°C ,65°C,67°C,70°C, 1 minute
Extension	72°C, 1minutes
Final extension	72°C, 10 minutes
Number of cycles	30

(Sambrook and Russel, Molecular Cloning, 2001)

4.7 MinElute Gel extraction of PCR product (Qiagen)

- PCR product was loaded onto low melting agarose gel and run.
- The gel was then excised where the bands for *deob* gene were visible under the UV illuminator by a sterile surgical scalpel.
- The excised gel pieces were placed in a clean microfuge tube which was previously weighed. The vial containing the gel slice was also weighed. Weight of excised gel was 150 mg. Binding buffer QG was then added accordingly (100 µl for every 100 mg) into the vial.
- Incubation at 50°C for 10 minutes was carried.3 gel volume QG buffer was added (450 µl). Vortexing was done every 2-3 minutes during incubation to completely dissolve agarose.
- After the gel slice has dissolved completely, it was checked that the color of mixture was yellow.
- 1 gel volume of isopropanol (450 µl) was added to the mixture and then mixed by inverting the tube.
- MinElute spin column was placed in provided 2 ml collection tube.
- Sample was applied to MinElute column and centrifugation was done at 12,000 rpm for 1 minutes. The flow through was discarded.
- 250 µl of buffer QG was added to MinElute column and centrifuged for 1 minute at 12,000 rpm. The flow through was discarded.
- 375 µl buffer PE was added to the column and centrifugation at 12,000 rpm for 1 minute was done. Flow through was discarded and MinElute column was placed back into same collection tube.
- Centrifuge column in a 2 ml collection tube was done for 1 min. Residual ethanol from PE buffer was not completely removed unless the flow through was discarded before the additional centrifugation.

- MinElute column was placed into a clean 1.5 ml microfuge tube. To elute DNA, 10 µl buffer EB was added to center of MinElute column. Column was allowed to stand for 1 min and then centrifugation was done at 12,000 rpm for 1 minutes to elute DNA.

- DNA was analyzed on 1% Low EEO agarose gel. The purified DNA was then stored at -20°C. This extracted product was then used for ligation.

(MinElute Gel Extraction Kit - Qiagen)

4.8 LIGATION (pTZ57R/T harbouring *deoB/cmk* gene)

For ligation, the following solutions were mixed (Thermo Scientific InsTAclone PCR Cloning Kit).

Ligation Mixture	Volume
Vector pTZ57R/T	3 µl (0.17 pmol ends)
5X Ligation Buffer	6 µl
PCR product	4 µl (0.52 pmol ends)
Water, nuclease-free	16µl
T4 DNA Ligase	1 µl
Total volume	30 µl

(Thermo Scientific InsTAclone PCR Cloning Kit).

The above mixture was vortexed briefly and centrifuged for 3-5 s. The ligation mixture was incubated at room temperature (22°C) for 1 hour. Then for the maximal number of transformants, the reaction was incubated overnight at 4°C.

4.9 TA cloning

2.5 µl of the ligation mixture was used directly for bacterial transformation. The day before the transformation, overnight culture was seeded by inoculating 2 ml

of C-medium with a single bacterial colony (not older than 10 days). Then the culture was incubated overnight at 37°C in a shaker.

On the day of transformation, LB agar plates supplemented with ampicillin (50µg/ml) were made. C-medium containing bottle was pre-warmed at 37°C for 20 minutes. 250 µl of T-solution (A) and 250 µl of T-solution (B) were mixed in a separate tube and kept on ice. 150 µl of the overnight bacterial culture was added to 1.5 ml of pre-warmed C-medium. Incubation was done for 20 min at 37°C in shaker. Bacterial cells were pelleted by 1 min centrifugation, and the supernatant was discarded. The cells were resuspended in 300 µl of T-solution and incubated on ice for 5 min. Then microcentrifugation was done for 1 min. Supernatant was discarded. Pelleted cells were resuspended in 120 µl of T-solution and incubated on ice for 5 minutes. Then 2.5 µl of ligation mixture (containing 14 ng vector DNA) was added into new microcentrifuge tubes and store on ice for 2 minutes. 50 µl of the prepared cells were added to each tube containing DNA, mixed and incubated on ice for 5 minutes. Plating was done immediately on pre-warmed LB-ampicillin agar plates and incubated overnight at 37°C.

4.10 Screening of transformants

Few of the *E.coli* DH5α transformant colonies were selected for further screening.

4.10.1 Plasmid isolation

The other half of the colonies were inoculated into LB broth supplemented with ampicillin (50µg/ml) and incubated overnight at 37˚C and plasmid was isolated from this culture using Alkaline Lysis method (Sambrook and Russel, Molecular Cloning, 2001). The isolated plasmid (pTZ57R/T harbouring *deoB* or *cmk* gene) and the expression vector (pET28a+) were then double digested with the restriction enzymes (Nde1 and Xba1).

Alkaline Lysis method

- Single colony (*E.coli* DH5α containing pTZ57R/T vector harbouring *deoB* or *cmk* gene) was inoculated in 5 ml of LB broth supplemented with ampicillin (50µg/ml) and incubated overnight at 37˚C on shaker. The culture was centrifuged at 12000 rpm for 5 min at 4˚C.

- Supernatant was discarded and the bacterial pellet was resuspended in 2 ml of culture and again microcentrifuged. Double pellet was done.

- The bacterial pellet was resuspended in 100 µl ice cold alkaline lysis solution I by vigorous vortexing and then 150 µl of alkaline solution II was added. The contents was mixed by inverting the tube rapidly five times and stored on ice. 400 µl of ice cold alkaline solution III was added in tube and mixed by inverting tube slowly till precipitates forms. The tubes were then stored on ice for 3-5 min and centrifuged at maximum speed for 10 minutes at 4˚C.

- Supernatant was transferred in fresh tube and equal volume of Phenol: Chloroform (1:1 v/v) was added and mixture was vortexed.

- The emulsion was centrifuged at max speed for 15 minutes at 4˚C. The aqueous layer was transferred to a fresh tube.

- 2 volume of 99% ethanol was added and the DNA was recovered by centrifuging at maximum speed for 15 minutes at room temperature.

- Supernatant was discarded and tubes were kept open at room temperature until the ethanol was evaporated.

- The pellet was dissolved in 20 µl of TE buffer and plasmid was stored at -20˚C.

(Sambrook and Russel, Molecular Cloning, 2001)

By gel extracting PCR product, dA overhangs were removed. So a fresh PCR was set up, which was done as mentioned above. Ligation mixture was made, as mentioned earlier. Bacterial transformation was performed by manually prepared competent cells.

4.11 Preparation of competent cells

- A single bacterial colony (2-3 mm in diameter) (from *E.coli*DH5α and BL21(DE3)) was picked from a plate and transferred into 5 ml LB broth in two different tubes.
- The culture was incubated for 12 hours at 37°C without shaking. 5 ml culture were then transfered into two different 100 ml LB broth flask. The culture was incubated for 3-4 hours at 37°C with vigorous shaking.
- The cells were recovered by centrifugation at 2700*g* (4100 rpm) for 10 minutes at 4°C. The supernatant was decanted and cell pellets were air dried. Each pellet was resuspended in 30 ml of ice-cold $CaCl_2$-glycerol solution.
- The cells were recovered by centrifugation at 2700*g* (4100 rpm) for 10 minutes at 4°C.
- The supernatant was discarded and the pellet was resuspended in 2 ml of ice-cold 0.1 M $CaCl_2$–glycerol solution.
- Now aliquotes of 50 µl competent cells was made. Store the competent cells at -80°C after flash freezing the cells with liquid nitrogen. The cells obtained were used for transformation.

(Sambrook and Russel, Molecular Cloning, 2001).

4.12 Transformation in *E.coli* DH5α

- To transform the $CaCl_2$ treated cells 50 µl *E.coli* DH5α suspension of competent cells was transferred to a sterile, chilled microfuge tube using a chilled micropipette tip.
- 10µl of the ligated product was added to the tube and contents were mixed by swirling gently. The tube was stored on ice for 15 minutes.

- Tube was trasnsferred to a rack placed in a preheated 42°C circulating water bath and kept for 90 seconds. The tube was transferred rapidly to an ice bath and allowed to chill for 2 minutes.

- 940 µl of LB medium was added to the tube. The culture was incubated for 45 minutes in a shaker set at 37°C to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid.

- 100 µl of transformed competent cells were transferred using spread plate method onto agar LB medium supplemented with appropriate antibiotic (50 µg/ml). The plate was stored at room temperature until the liquid had been absorbed.

- The plate was inverted and incubated at 37°C. Transformed colonies appeared in 12-16 hours.

(Sambrook and Russel, Molecular Cloning ,2001)

4.13 Plasmid isolation of transformants (TA Clones)

Plasmid DNA Mini kit-Qiagen

- 20 ml of LB Broth supplemented with the antibiotic (50 µg/ml) was inoculated with a single colony (*E.coli* DH5α containing pTZ57R/T vector harbouring *deob/cmk* gene) and incubated overnight at 37°C with vigorous shaking.

- 2 ml of bacterial culture was taken in a 2 ml vial and centrifuged at maximum speed for 2 minutes.

- Supernatant was discarded and again 2 ml of bacterial culture was added to double down the pellet.

- Supernatant was discarded and 100 µl of solution I was mixed and was allowed to stand for a minute. Then 200 µl of solution II was added, mixed by inverting the tube 4-6 times and was allowed to stand for a minute at room temperature. Then 350 µl of Solution III was added and mixed by inverting the tube and was allowed to stand for 1 minute at room temperature.

- The tubes were then centrifuged at 12000 rpm for 5 minutes.

- The supernatant was transferred to MX-10 column and centrifuged for 2 minutes at 10,000 rpm. The flow through was discarded and 500 µl of wash solution was added to the column and centrifuged at 10,000 rpm for 2 minutes. The washing step was repeated. The flow through was discarded and centrifugation was done for an additional 1 minute to remove the remaining wash solution if any.

- Then the column was transferred to a new vial and 50 µl of elution buffer was added into the center part of the column and incubated for 2 minutes at room temperature.

- Centrifugation was done for 2 minutes at 10,000 rpm to elute the plasmid. The elution step was repeated and the isolated plasmid was stored at -20°C.

4.14 Restriction Enzyme digestion

The two plasmids (recombinant vector and expression vector) were restricted using the same conditions and enzymes i.e. Nde I and Xba I.

In two sterile microfuge tubes, amplified gene (*deoB* gene) and expression vector (pET28a+) were double digested with the two restriction enzymes (NdeI and XbaI). Solutions were added in the following order:

Restriction digestion mixture	**Volume**
10X Restriction enzyme buffer	2 µl
Sterile distilled water	13.5 µl
BSA	0.5 µl (100 µg/ml)
Plasmid (amplified gene)	3 µl (1 µg)
Restriction enzyme (NdeI)	0.5 µl (10 U)
Total	19.5 µl

After mixing all the solutions properly, the reaction mixture was incubated at 37°C in a water bath for 1 hour. The restriction enzyme was heat inactivated by incubating the tube at 65°C for 15 minutes.

Then, 0.5 µl (10 U) Restriction enzyme (XbaI) was added to the above reaction mixture and incubated at 37°C in a water bath for 1 hour. The restriction enzyme was heat inactivated by incubating the tube at 65°C for 15 minutes.

The same protocol was used for restricting the expression vector pET28a^{+} (Ausubel, 2002). Then the gene and the vector restricted with the same restriction enzymes were ligated.

The ligated DNA product was then transformed into competent *E. coli* DH5α cells on LB supplemented with Kanamycin (50µg/ml).

4.15 Extraction of restricted fragments from Agarose gel

- The restricted vector (pTZ57R/T) with the insert was loaded onto low melting agarose gel and run. The restricted pET28a$^+$ was also loaded onto the gel.
- The gel was then excised where the bands for insert and restricted pET28a$^+$ were visible under the UV illuminator by a sterile surgical scalpel.
- The excised gel pieces were placed in a clean microfuge tube which was previously weighed. Both the insert and the pET28a$^+$ were placed in a separate vial. The vial containing the gel slice was also weighed.
- Binding buffer was then added accordingly (400 µl for every 100 mg) into the vial and incubated at 50°C for 10 minutes. Occasional shaking was done to completely dissolve agarose.
- The above mixture was transferred to the MX-10 column and was allowed to stand for 2 minutes. Centrifugation was done at 10,000 rpm for 2 minutes. The flow through was discarded.
- Then 500 µl of wash solution was added and centrifuged for 1 minute at 10,000 rpm. The flow through was discarded. The washing step was again repeated and centrifugation at 10,000 rpm for additional 1 minute was done to remove any residual wash solution.
- Then the column was placed in a clean 2 ml microfuge tube and 30-50 µl of elution buffer was added to the center part of the column and incubated for 2 minutes at room temperature. Centrifugation was done at 10,000 rpm for 2 minutes to elute DNA. The purified DNA was then stored at -20°C.
- These extracted restriction products were then used for ligation.

4.16 Ligation of vector and insert

In a sterile microfuge tube, solutions were added in the following order

Ligation Mixture	Volume
10X Ligation Buffer	2 µl
Sterile deionized water	11 µl
Restricted pET28a^{+}	4 µl (50 ng)
Restricted Insert (*deoB*gene)	12 µl (50 ng)
T4 DNA Ligase	1 µl
Total volume	30 µl

These reaction mixtures were gently mixed and incubated at 22˚C for an hour and then incubated at 4˚C for overnight (Ausubel, 2002).

The ligated DNA product was then transformed into competent *E. coli* DH5α cells on LB-Kanamycin plates supplemented with kanamycin concentration 50µg/ml. Here transformation was performed as mentioned earlier.

4.17 Screening of transformed colonies

Screening of recombinant plasmid in the transformed cells was done by plasmid isolation.

(Sambrook and Russel, Molecular Cloning, 2001)

5. RESULTS

5.1 Isolation of the Genomic DNA from *Thermus thermophilus*

Genomic DNA from *Thermus thermophilus* was isolated using the standard protocol given in Short Protocols in Molecular Biology, Edition 4, Volume 1 (fig. 5.1– Lanes A, B and C). The genome size of *Thermus thermophilus* is 2.12 Million bp. It was analyzed on 0.8 % low EEO (Electroendoosmosis) agarose gel as shown in fig. 5.1. After the genomic DNA isolation, there was too much RNA contamination, which can interfere in PCR reactions to be followed, so RNase treatment was given (fig. 5.2).

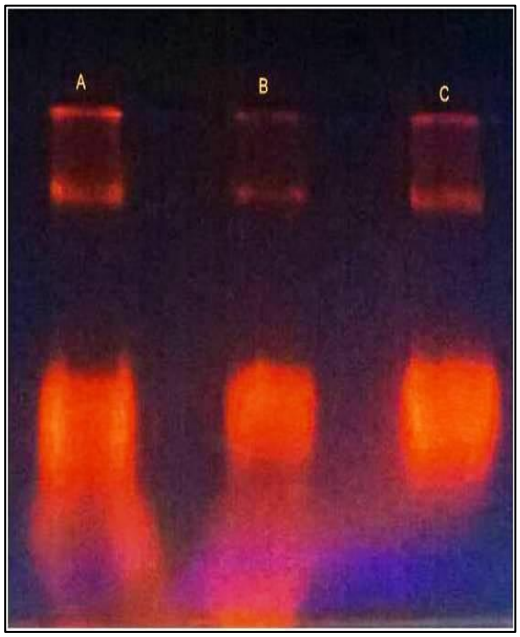

Fig. 5.1 : 0.8 % Low EEO Agarose gel electrophoresis of isolated genomic DNA from *Thermus thermophilus*.

Lane A,B,C : Genomic DNA before RNase treatment.

5.2 RNase treatment: RNA was removed by treatment with RNase.

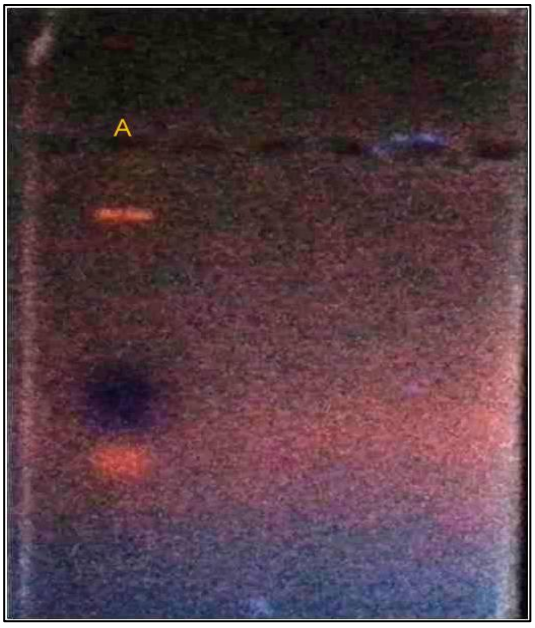

Fig. 5.2: 0.8% Low EEO Agarose gel electrophoresis of genomic DNA after RNase treatment.

Lane A : Genomic DNA after RNase treatment.

5.3 Amplification of desired gene (*deoB*) by Polymerase Chain Reaction

PCR amplification of phosphopentomutase (*deoB*) gene was carried out using primers from the gene sequence. The optimum PCR conditions was first established for the efficient amplification of the *deoB* and then subsequently used for amplification. The amplified product for *deoB* gene was analyzed on 1% Low EEO agarose gel fig-5.3. A band at 1.15 kbp corresponding to the correct size of the *deoB* gene was obtained.

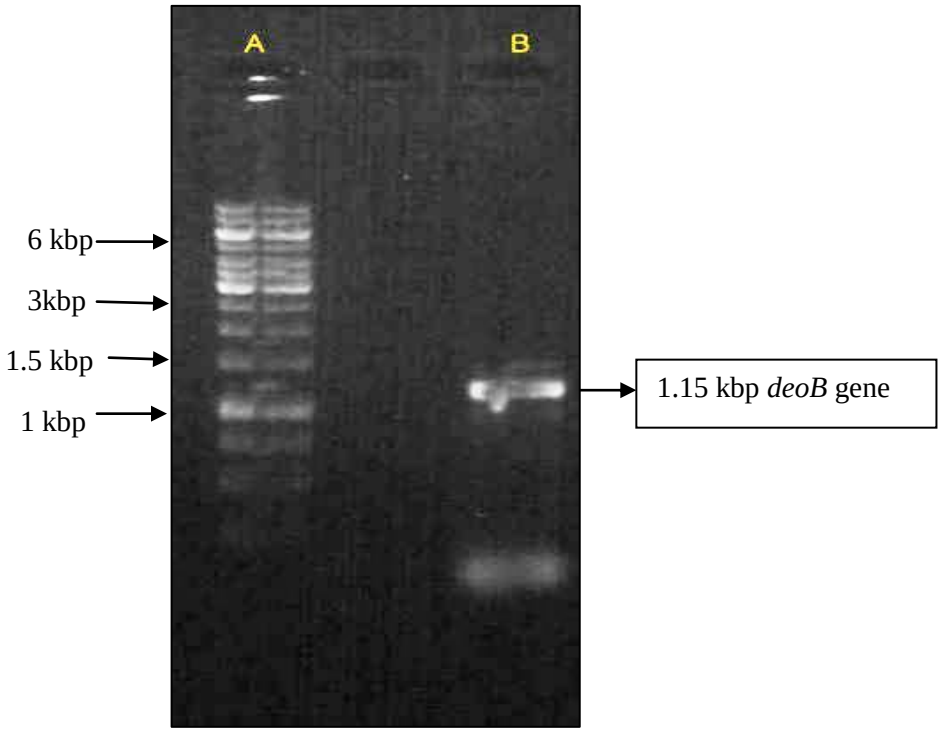

Fig 5.3: 1% Low EEO Agarose gel electrophoresis image of PCR amplified *deoB* gene.

Lane 1: **GeneRuler™** 1kb DNA ladder (ThermoScientific).

Lane 3: PCR Product.

5.4. Gel extraction of PCR product (*deoB* gene)

Amplified product of *deoB* gene was gel extracted and purified using Minelute Gel Extraction kit (Qiagen). For gel extraction of unpurified PCR product 1% low melting agarose gel was used. The purified amplified product i.e. *deoB* gene was analyzed on 1% Low EEO agarose gel as shown in fig 5.4. Purified PCR Product (*deoB*) corresponding to the size of (1.15 kbp) was seen.

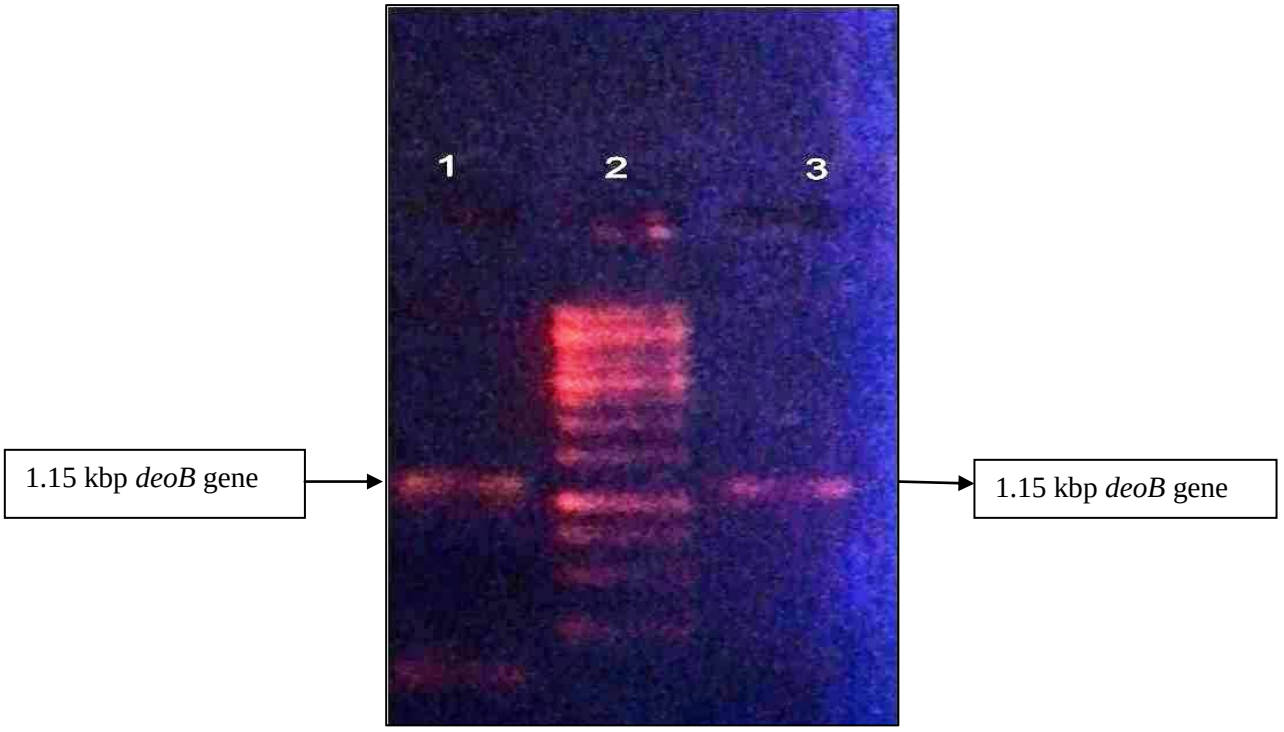

Fig. 5.4: 1% Low EEO Agarose gel electrophoresis of purified PCR Product.

Lane 1 : Unpurified PCR Product (*deoB).*

Lane 2 : GeneRuler™ 1kb DNA ladder (Thermoscientific) .

Lane 3 : Purified PCR Product (*deoB).*

5.5 Cloning of *deoB* gene in pTZ57R/T plasmid

The purified PCR product corresponding to the 1150 bp *deoB* gene was then cloned into the pTZ57R/T vector using the poly dT and poly dA overhangs of the vector and fresh PCR product respectively. This was carried out using Thermo Scientific InsTAclone PCR Cloning Kit. The ligated product was analyzed on a 1 % agarose gel. No bands corresponding to the expected 4.03 kbp of the ligated product was observed fig 5.5(a), indicating that ligation has not worked and the gene has not been cloned into pTZ57R/T vector. This could be due to loss of poly dA overhangs of the PCR product during gel purification. Hence cloning into pTZ57R/T vector was repeated using unpurified PCR product. This time a band corresponding to 4.03 kbp was obtained as shown in fig 5.5(b).

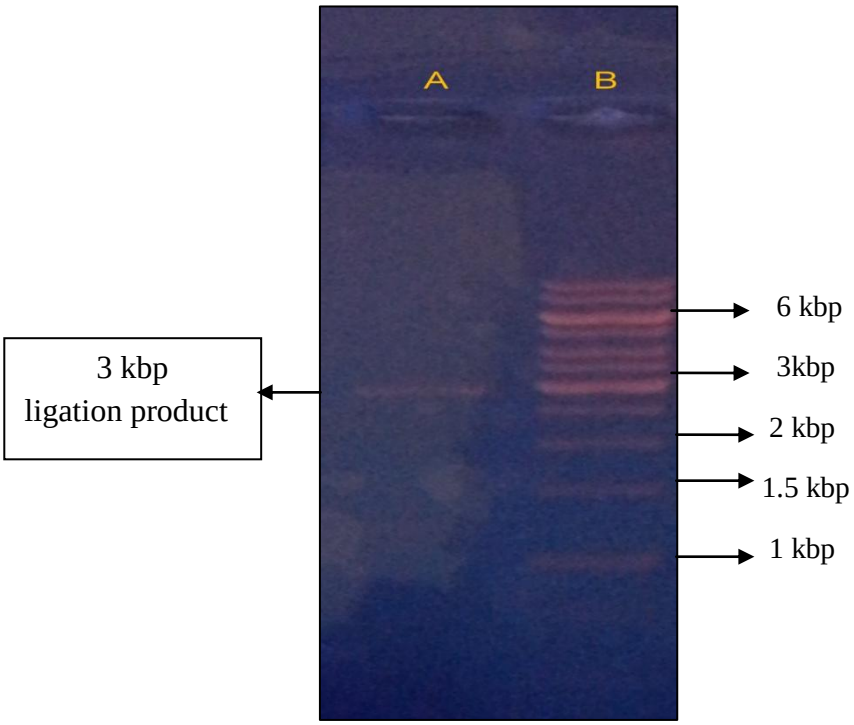

Fig. 5.5(a): 1% Agarose gel electrophoresis analysis of ligation mixture product.

Lane A: Ligation mixture.

Lane B: GeneRuler™ 1kb DNA ladder (ThermoScientific).

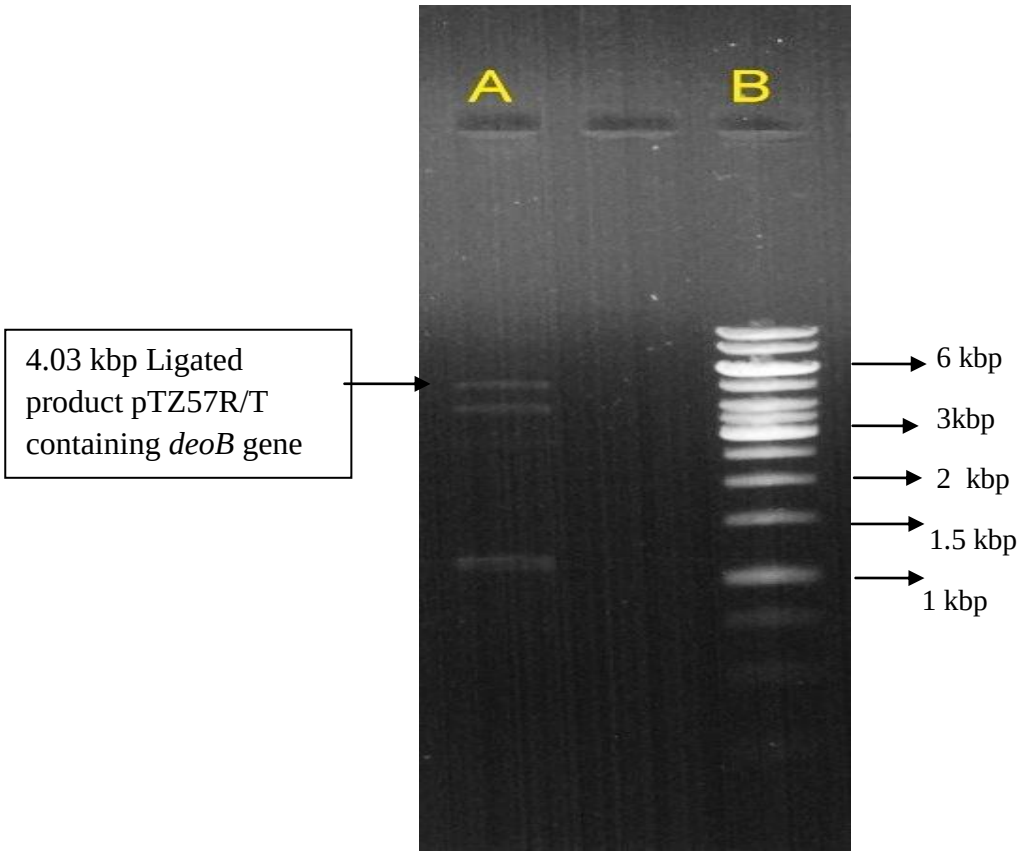

Fig. 5.5(b) : 0.8% Low EEO Agarose gel electrophoresis for analysis of ligated product.

Lane A: Ligated product.

Lane B: GeneRuler™ 1kb DNA ladder (ThermoScientific).

5.6 Isolation of recombinant TA plasmid containing *deoB* gene from *E.coli* DH5α cells

The ligated product that was at the correct size was transformed into *E.coli* DH5α cells. The plasmid pTZ57R/T harbouring the *deoB* gene was then extracted and purified from the transformants using the Qiagen Plasmid DNA Mini kit. Several transformant colonies were picked out to confirm the positive clone. The purified plasmids were analyzed on 1 % agarose gel as shown in fig 5.6. The correct sized plasmid of 4.03 kbp was obtained. It was further confirmed by insert release.

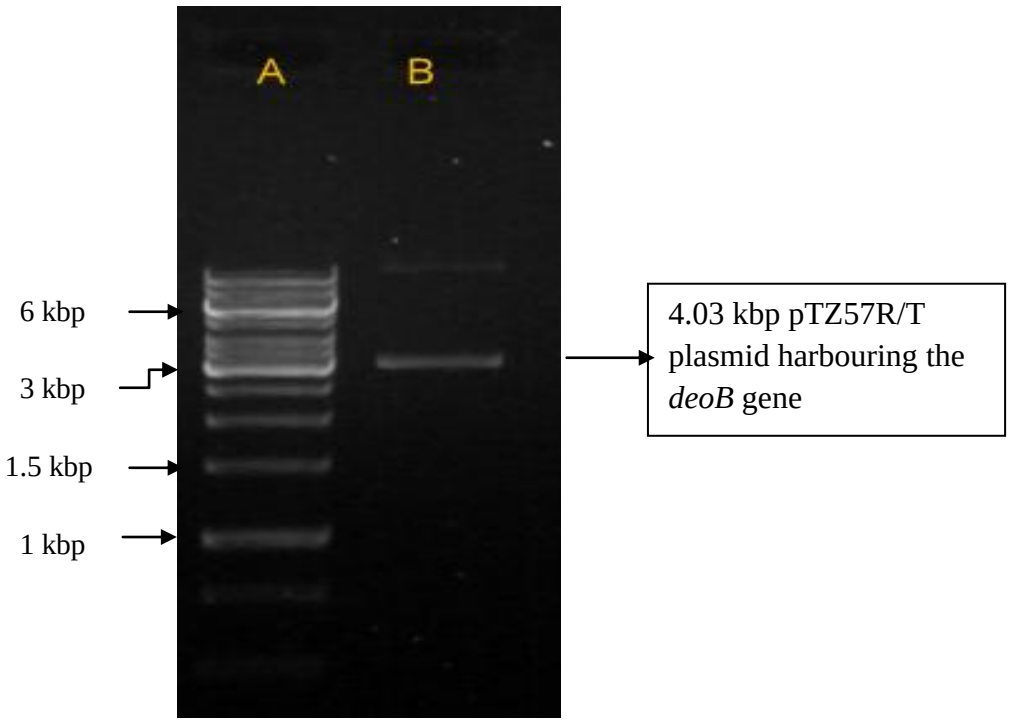

Fig.5.6: 1% Agarose gel electrophoresis showing the recombinant TA vector having the gene of interest

(*deoB*) after transformation, followed by plasmid isolation.

Lane A : GeneRuler™ 1kb DNA ladder (ThermoScientific).

Lane B : The TA vector containing the *deoB* gene that was isolated after transformation in DH5α cells.

5.7 Confirmation of clones by insert release

The clones were confirmed by release of a 1.15 kbp insert fragment after double digestion of the recombinant TA plasmid by NdeI and XbaI. The insert release from the various clones are shown in fig (5.7). Thus the positive clones werw confirmed. These double digested *deoB* gene fragment was then subcloned into the expression vector, pET28a^{+} .

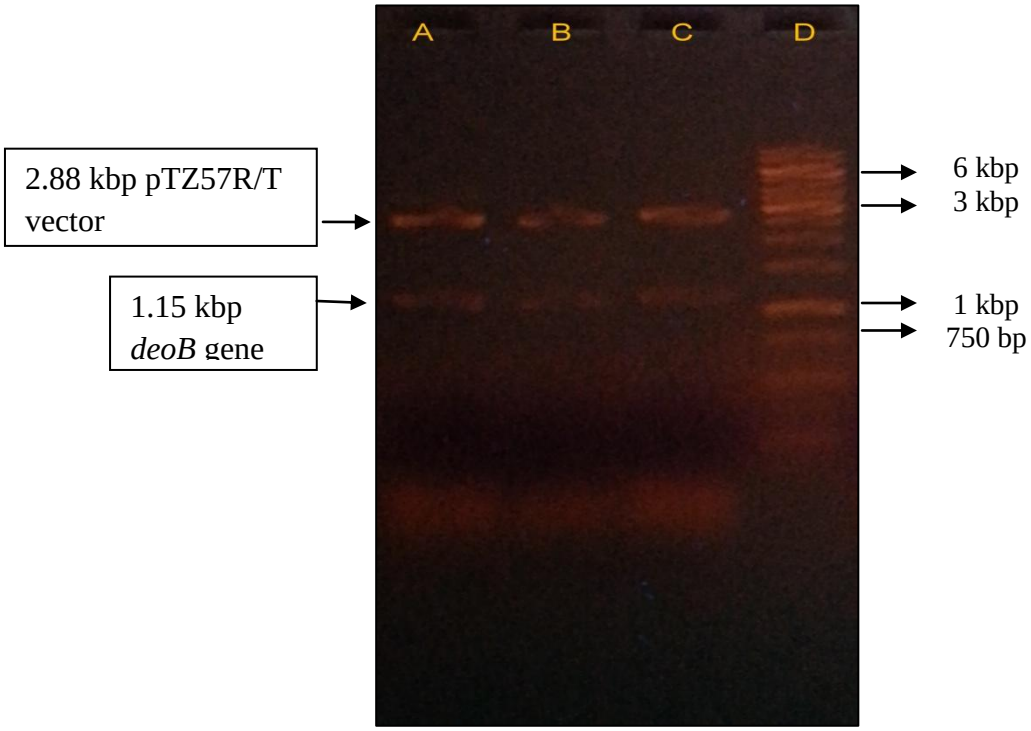

Fig.5.7: 1% Low EEO Agarose gel electrophoresis showing the double digested product of the pTZ57R/T harbouring *deoB* gene by the restriction enzymes NdeI and XbaI.

Lane A, B and C: The released *deoB* gene after double digestion of the recombinant TA vector from three different clones.

Lane D: GeneRuler™ 1kb DNA ladder (ThermoScientific).

5.8 Isolation & purification of expression vector pET28a$^+$

Isolation and purification of pET28a$^+$ plasmid was done by Qiagen Plasmid DNA Mini kit. Fig.5.8 shows the band for the isolated plasmid (pET28a$^+$) which is of size 5.3 kbp.

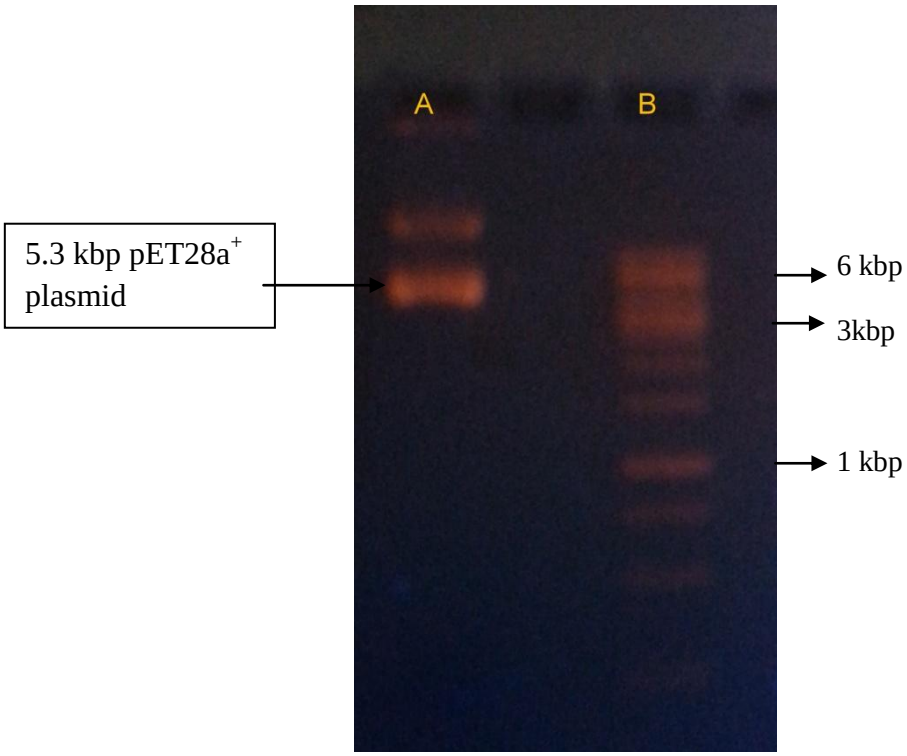

Fig.5.8: 0.8 % Low EEO Agarose gel electrophoresis showing the size of pET28a$^+$

Lane A: pET28a$^+$ plasmid.

Lane B: GeneRuler™ 1kb DNA ladder (ThermoScientific).

5.9 Ligation of the *deoB* gene and the double digested pET28a$^+$ vector

The purified pET28a$^+$ vector was double digested by the restriction enzymes NdeI and XbaI & was ligated to the 1.15 kbp *deoB* gene fragment released by double digestion of the TA clone by the same enzymes. T4 DNA ligase was used for ligation. The ligated product was analyzed on a 1% agarose gel & it is shown in fig.(5.9). No band was seen at the expected size of 6.45 kbp indicating that the ligation had failed.

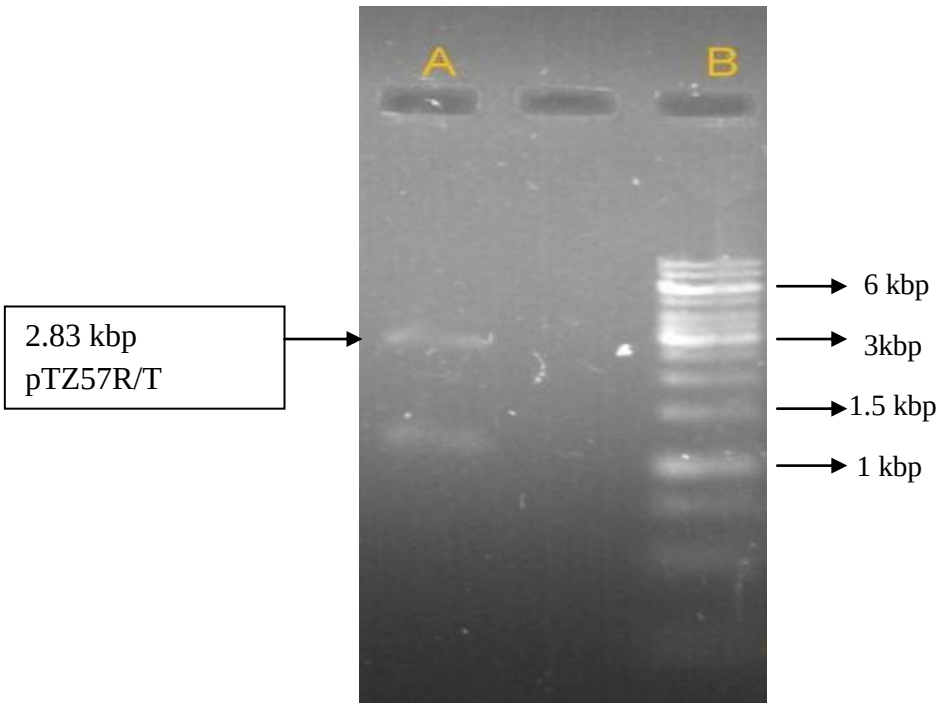

Fig.5.9: 1% Low EEO agarose gel electrophoresis showing ligation of the pET28a$^+$ and *deoB* gene.

Lane A: Ligation mixture

Lane C: GeneRuler™ 1kb DNA ladder (ThermoScientific)

5.10 Amplification of desired gene (*cmk*) by Polymerase Chain Reaction

Gradient PCR was setup for the amplification of *cmk* gene at the annealing temperatures 55 °C, 60°C, 63°C, 65°C, 67°C and 70°C for 1 minute. The amplified product was analyzed on 1% Low EEO Agarose gel (fig-5.10). The bands corresponds to the *cmk* gene (0.627 kbp) obtained after PCR amplification.

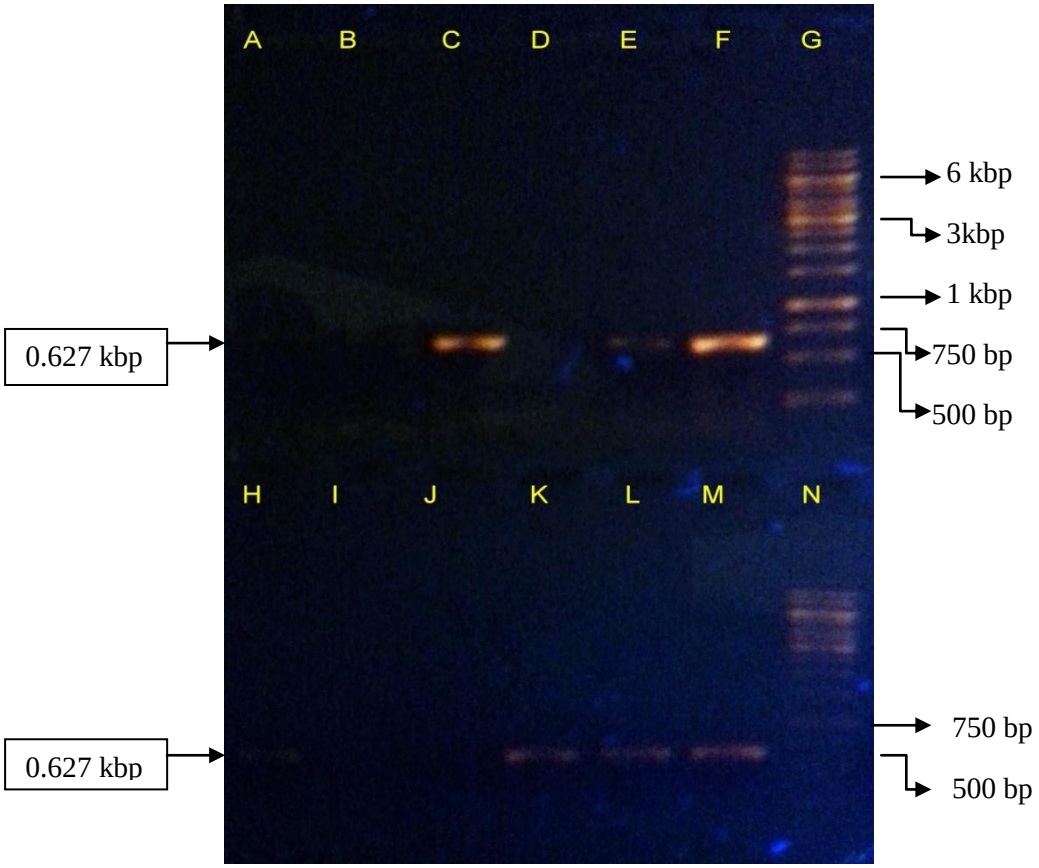

Fig. 5.10: 1 % Low EEO Agarose gel electrophoresis image of PCR amplified *cmk* gene.

Lane G and N: **GeneRuler™** 1kb DNA ladder (ThermoScientific).

Lanes A, B, C, D, E, F, H, I, J, K, L, M : PCR Products.

Lanes A & B (55°C), Lane C & D (60°C), Lane E anf F (63°C), Lane H & I (65°C), Lane J & K (67°C) & Lane L & M (70°C).

Note: Amplification of *cmk* gene from *Thermus thermophilus* was standardized on 63°C.

5.11 Cloning of *cmk* gene in pTZ57R/T plasmid

The gene encoding cytidylate kinase from *Thermus thermophilus* was amplified by PCR, using manually designed primers. Using genomic DNA isolated from *Thermus thermophilus* as template, a 627 bp fragment that corresponding to full length of *cmk* gene was obtained. This PCR product having dA overhangs was ligated to the pTZ57R/T vector which has complementary dT overhangs by using Thermo Scientific InsTAclone PCR Cloning Kit (fig.5.11).

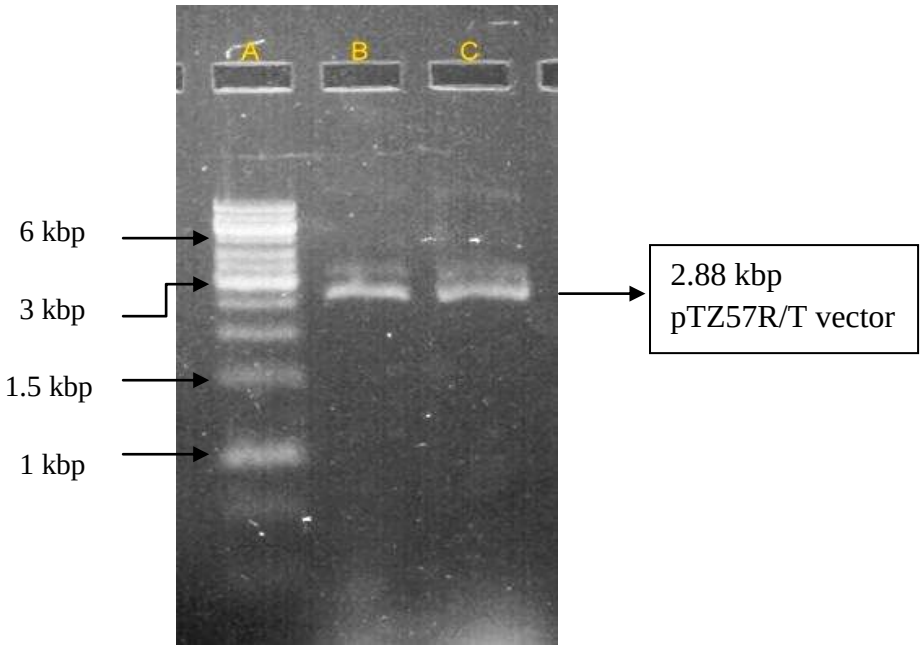

Fig.5.11: 0.8% Low EEO Agarose gel electrophoresis showing the recombinant TA vector having the gene of interest (*cmk*).

Lane A: GeneRuler™ 1kb DNA ladder (ThermoScientific).

Lane B and C: Ligated product

6. DISCUSSION

Proteins spontaneously fold up into three-dimensional structures that are determined by the sequence of amino acids in the protein polymer. Extremophilic proteins tend to be more stable, and are easier to crystallize than the mesophilic proteins. Extremophilic enzymes have become model systems to study enzyme evolution, enzyme stability and activity mechanisms, protein structure–function relationships and biocatalysis under extreme conditions. Enzyme-catalyzed phosphoryl transfer forms the basis for many biological, bioenergetic and regulatory processes and is one of the most common cellular reactions. Phosphomutases are phosphotransfer enzymes that rearrange the position of phosphate within a substrate molecule through either intramolecular or intermolecular phosphoryl transfer. Cytidylate kinase (*cmk*) is a member of the nucleoside monophosphate kinase (NMK) family. These two enzymes play an important role in biosynthetic and metabolic pathway.

TA cloning is one of the simplest and most efficient methods for the cloning of PCR products. The use of a linearized "T-vector" which has single 3'-T overhangs on both ends allows direct, high-efficiency cloning of PCR products, facilitated by complementarity between the PCR product 3'-A overhangs and vector 3'-T overhangs.

Double digesting the TA vector having the insert with the restricting enzymes and ligating the gene insert into the expression vector was the crucial step in our gene cloning experiment. Purification of the insert after double digestion of our recombinant TA vector and also purification of double digested pET28a^{+} was done.

As both our vectors i.e. recombinant vector and Expression vector (pET28a^{+}) have the different antibiotic resistant genes (Ampicillin and Kanamycin respectively), selection procedure was easy. But the purified product i.e. insert and vector, when ligated, was unsuccessful as seen on

agarose gel. Hence expression of the protein was not checked for. There could be many reasons for unsuccessful ligation. Troubleshooting was done.

Thus ligation of recombinant vector and expression vector will be done again and further expression and purification will be performed.

The two enzymes Phosphopentomutase and Cytidylate kinase can further be engineered to be used as novel antibiotic target for the organisms *Francisella tularensis* and *Streptococcus pneumoniae* respectively. Thus with an increasing market for the enzymes, leading to production in higher volumes, the cost is however predicted to decrease. Moreover, with a paradigm shift in industry moving from fossils towards renewable resource utilization, the need of microbial catalysts is predicted to increase, and certainly there will be a continued and increased need of thermostable selective biocatalysts in the future.

7. APPENDIX

1. TE Buffer: (Tris-EDTA)
   10mM Tris-Cl (pH 7.4, 7.5 or 8.0)
   1mM EDTA (pH 8.0)

2. CTAB/NaCl Solution
   Dissolve 4.1g NaCl in 80 ml water and with stirring, slowly add 10 g CTAB (Cetyltrimethyl ammonium bromide). If necessary heat to 65˚C.
   Adjust volume to 100 ml.

3. Alkaline Lysis Solution I
   50mM Glucose
   25mM Tris-Cl (pH 8)
   10mM EDTA (pH 8)

4. Alkaline Lysis Solution II
   0.2N NaOH (prepared freshly from 10N NaOH)
   10 % SDS

5. Alkaline Lysis Solution III
   5M Potassium Acetate 60ml
   Glacial Acetic Acid 11.5ml
   Water 28.5ml

6. TAE Buffer: (50X, per liter)
   242g of Tris Base
   57.1g of glacial acetic acid
   100ml of 0.5M EDTA (pH 8.0)

7. 6X Agarose Gel Loading Buffer

   0.25% (w/v) bromophenol blue

   0.25% (w/v) Xylene cyanol FF

   30% glycerol in water

8. 0.5M EDTA (pH 8.0)

   Add 186.1g of EDTA to 800ml of water. Stir well and adjust pH to 8 using NaOH.

9. *Thermus thermophilus* medium MTCC no. 1496 (TBP broth)

   Beef extract : 4g

   Polypeptone : 4g

   K_2HPO_4 : 3g

   KH_2PO_4 : 1g

   Agar : 25g

   Distilled water : 1 liter

   Adjust pH to 7.0

10. Luria-Bertani Broth,Miller

    Casein hydrolysate : 10 g/l

    Yeast extract : 5 g/l

    Sodium Chloride : 10 g/l

11. Luria-Bertani Agar medium

    Casein hydrolysate : 10 g/l

    Yeast extract : 5 g/l

    Sodium Chloride : 10 g/l

    Agar : 15 g/l

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