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Expression of Taq Polymerase I Gene in *Escherichia coli* BL21

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ABSTRACT

The thermostable properties of Taq DNA polymerase from *Thermus aquaticus* have contributed greatly to the yield, specificity, automation, and utility of the polymerase chain reaction method for amplifying DNA. Taq polymerase is widely used enzyme for DNA amplification in PCR techniques and highly applicable in molecular biology and biotechnology. In this study the Taq gene was amplified from the genomic DNA of the organism *Thermus aquaticus* and cloned into pET100 Directional TOPO® vector. The *Escherichia coli* BL21 were transformed using the recombinant pET100 (8.3 Kb) containing the gene of interest and the clones of right orientation were selected and followed by protein induction. Expression of gene was observed on SDS PAGE. The band observed around 94 KDa for Taq Pol I. It is feasible, that Taq with a higher fidelity rate could be developed. The challenge is fulfillment of rising demand of Taq DNA polymerase variants that add modified nucleotides for efficient DNA amplification and labeling, this challenge can be answered by using the techniques available in protein engineering like random mutagenesis and site directed mutagenesis which will create various variants of existing Taq polymerase and screening to find out the best one which fits the above necessities.

Key words: *Thermus aquaticus*, Taq polymerase, expression, Purification.

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INTRODUCTION

DNA polymerase plays a leading role in the replication and maintenances of the genome and are central to the accurate transmission of genetic information from generation to generation that is presented in all living cells. Thermostable DNA polymerase is a very important enzyme for molecular biological studies such as DNA amplification and DNA sequencing by the PCR [1, 2]. Most of the thermostable DNA polymerases have been isolated from a heat stable bacterium called *T. aquaticus* having a molecular weight of about 6.6×10^4–9.4×10^4 Daltons [3,4]. *T. aquaticus* is a bacterium that lives in hot springs and hydrothermal vents [5]. Taq polymerase was identified as an enzyme able to withstand the protein-denaturing conditions [high temperature] required during PCR [6]. Therefore it replaced the DNA polymerase from *E. coli* originally used in PCR [7]. Taq's temperature optimum for activity is 75-80^{0}C, with a half-life of 9 minutes at 97.5^{0}C, and can replicate a 10^3 base pair strand of DNA in less than 10 seconds at 72^{0}C [8].

The incorporation of dNTPs into DNA is catalyzed by Taq DNA polymerase I. It requires a DNA template, a primer terminus, and the divalent cation Mg^{2+}. Taq polymerase contains a polymerization dependent 5'-3' exonuclease activity. It does not have a 3'-5' exonuclease and thus no proof reading function. Despite this, the enzyme synthesizes DNA In vitro with reasonable fidelity [3]. Use of the thermostable Taq polymerase eliminates the need for having to add new enzyme to the PCR reaction during the thermocycling process. A single closed tube in a relatively simple machine can be used to carry out the entire process. Thus, the use of Taq polymerase was the key idea that made PCR applicable to a large variety of molecular biology problems concerning DNA analysis [6].

The Taq DNA polymerase expressed in *E. coli* shows identical characteristics to native Taq from *T. aquaticus* with respect to activity, specificity, thermostability and performance in PCR [9]. However, the lac promoter and its derivatives are widely employed for the purposes mentioned above, and in most cases, IPTG is used as inducer for foreign gene expression [5, 6].

Taq polymerase was described as being a low fidelity enzyme [error rate between 1×10^{-4} and 2×10^{-5} errors per base pair, depending on experimental conditions] [8]. Yet, one should keep in mind that this corresponds to a quite good accuracy [inverse of the error rate] since 45,000 nucleotides [nt] can be incorporated into newly synthesized DNA strands before an error occurs. Like other DNA polymerases lacking 3′→5′ exonuclease activity, Taq polymerase exhibits a deoxynucleotidyl transferase activity that is accountable for the addition of a few adenine residues at the 3′-end of PCR products.

The original PCR technique was slow and labor intensive because fresh DNA polymerase had to be added after every heat denaturation step. An important modification of the original PCR technique was the substitution of Taq DNA polymerase in place of the Klenow fragment [KF] of *E. coli* DNA polymerase I [5]. The PCR technique using Taq DNA polymerase was patented by Cetus Corporation in 1989 and the patent rights were later sold to the pharmaceutical company Hoffmann-La Roche for $300 million. In 1989 Science magazine

named Taq DNA polymerase the "Molecule of the Year", and in 1993, Kary Mullis was awarded the Nobel Prize in Chemistry for developing PCR. Currently the market for Taq DNA polymerase is in the hundreds of millions of dollars per year.

Taq DNA polymerase has become the standard reagent for the PCR reaction. The Taq gene cloned and used to produce the enzyme in nonthermophilic host bacteria, so both native Taq, isolated from *T. aquaticus*, and cloned Taq, isolated from expression systems in other bacteria, are commercially available. In addition, a number of other thermostable DNA polymerases, isolated from other thermophilic species, have become available. Among these are enzymes from Pyrococcus furiosus [Pfu polymerase], Thermus thermophilus [Tth polymerase], Thermus flavus [Tfl polymerase], Thermococcus litoralis [Tli polymerase aka Vent polymerase], and Pyrococcus species GB-D [Deep Vent polymerase]. Each of these, and other, polymerases has a specific set of attributes that can be selected depending upon the application. In general, there are three aspects of a DNA polymerase that should be considered. These are: 1-Processivity, 2-Fidelity and 3-Persistence.

Processivity refers to the rate at which that polymerase enzyme makes the complementary copy of the template. The standard here is Taq polymerase, which has a processivity of 50-60 nt per second at 72^0C. The rule of thumb is too simply assume a low-end default processivity for Taq polymerase of 1000 nt per minute bearing in mind that setting your extension times for this assumed value is more than adequate. Many of the other polymerases listed above are slower than Taq. For example, Tth polymerase processivity is on the order of 25 nt per second and others such as Vent and Deep Vent fall in this range as well. However, these enzymes have advantages over Taq polymerase that derive from and compensate for lower processivity. One of the most important of these other features is fidelity. This refers to the accuracy of the complementary copy being made. Taq DNA polymerase has among the highest error rates of the thermophilic polymerases at 285 x 10^{-6} errors per template nucleotide. Tli polymerase has a proof reading ability that is five-fold better than Taq at 57 x 10^{-6} errors per template nucleotide and Pfu polymerase also demonstrates fidelity in this range [10]. Finally, the attribute of persistence, which refers to the stability of the enzyme at high temperature, is intimately linked to the other two polymerase attributes. Stability can be measured in terms of how long the enzyme retains at least one-half of its activity during sustained exposure to high temperature. Taq polymerase has a half-life of about an hour and a half at a sustained 95^0C. Other enzymes have much longer half-life. Tli polymerase has a half-life of over six hours and Deep Vent polymerase has shown a half-life of nearly a full day when exposed to a constant 95^0C.

With the increased demand of Taq pol I for the research purpose there is increase in load on economics of the research laboratory. This economic load has generated the idea to develop Taq pol I in the labs at local level for the reduction of research cost, but the culture of *T. aquaticus* and isolation and purification of Taq pol I is again challenging. Keeping this view in mind the present study were done to developed a clone of Taq pol I in lab which was use for expression of gene and production of Taq pol I enzyme.

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MATERIALS AND METHODS

Bacterial strains:

Thermus aquaticus [MTCC-1494] supplied by IMTECH Chandigarh. *E. coli* strain BL21 and plasmid pET was purchased from Invitrogen [Champion™ pET100 Directional TOPO® Expression Kit with BL21 Star™ [DE3]].

Media Used:

a) TYP Media: TYP growth medium is used for the reviving of the cell of lyophilized *T. aquaticus* [MTCC-1494] supplied by IMTECH Chandigarh.

b) Luria Bertani [LB] Media: *E. coli* strains Star™ [DE3] were grown at 37^0C in LB broth and plated on LB agar containing 80 µg/ml ampicillin as described by Sambrook [8]. LB a nutritionally rich medium was developed for the growth and maintenance of pure cultures of bacterial strains. Tryptone provides peptides and peptones. Yeast Extract provides vitamins and certain trace elements. Ions for transport and osmotic balance are provided by NaCl. The prepared LB powder was supplied by laboratory. LB broth was prepared by dissolving LB powder at a concentration of 2.5 %. Agar at a concentration of 1.5 % was added for making LB agar media.

Genomic DNA isolation and PCR:

The Genomic DNA of *T. aquaticus* was isolated using high pure PCR template preparation kit, which was purchased from Roche Co. [Germany]. The isolated DNA sequenced was amplified by using thermocycler machine which follows the mechanism of polymerase chain reaction [PCR].

PCR involves sequence cycles, composed of three steps:

a. Denaturation of template DNA typically at about 94^0C.
b. Annealing of primers to the template at temperature 54^0C.
c. Extension of primers at about 72^0C.

The master mix was prepared in 1.5 ml eppendorf tubes as per the composition given in the following Table 1.

The master mix of above components was taken in microfuge tubes and finally added Taq Polymerase enzyme to start reaction. Now the microfuge tubes were placed in PCR machine. The thermocycler was programmed to run according to the conditions mentioned Table 2.

ISSN: 0975-8585

Table 1: Polymerase chain reaction mixture

INGREDIENTS	VOLUME
Buffer	5.0 µl
Forward Primer 5' - CGGAATTCTGAGGAGGTAACATGAGGG -3'	0.5 µl
Reverse Primer 5'-CGTCGACTAGATCACTCCTTGGCGGAGAG -3'	0.5 µl
dNTP's	2.0 µl
DNA	10.0 µl
Taq Polymerase	1.0 µl
Distilled Water	28.5 µl
TOTAL	**50.0 µl**

Table 2: Conditions for polymerase chain reaction cycles

Cycle	Steps	Temperature and Duration
Cycle 1 (1X)	Step 1	94^0C for 1 min
Cycle 2 (30X)	Step 1	94^0C for 1 min
	Step 2	52^0C for 30 sec
	Step 3	72^0C for 90 sec
Cycle 3 (1X)	Step 1	72^0C for 90 sec

Elutions of Taq DNA form agarose gel and its ligation:

The elution of DNA fragments from agarose gels was done by a kit [Agarose Gel DNA Extraction Kit] purchased from Roche Co. [Germany] and for ligation pET100 Directional TOPO® Vector were used. pET100 Directional TOPO® Vector is a bacterial plasmid designed to enable the quick production of a large quantity of any desired protein when activated [11]. This plasmid contains several important elements such as a lacI gene which codes for the lac repressor protein, a T7 promoter which is specific to only T7 RNA polymerase [not bacterial RNA polymerase] and also does not occur anywhere in the prokaryotic genome, a lac operator which can block transcription, a polylinker, and f1 origin of replication [so that a single-stranded plasmid can be produced when co-infected with M13 helper phage], an ampicillin resistance gene, and a pBR322 origin of replication. The restriction enzymes EcoRI and SalI had used to cut the plasmid [pET] open. DNA ligase was used to ligate the DNA fragments into the pET.

Protein induction:

Control of the pET expression system is accomplished through the lac promoter and operator [12]. Before target gene can be transcribed, T7 polymerase must be present. The gene on the host cell chromosome usually has an inducible promoter which is activated by addition of Isopropyl-beta-thio galactopyranoside [IPTG] [11]. This molecule, IPTG, displaces the repressor from the lac operator. Since there are lac operators on both the gene encoding T7 polymerase and target gene, IPTG activates both genes. Therefore, when IPTG is added to the cell, the T7 polymerase is expressed, and quickly begins to transcribe target gene which is then

translated as desired protein. IPTG works to displace a lac repressor since IPTG is an analogue of lactose. The lac genes express enzymes which are involved in the breaking down of lactose, and therefore, the presence of lactose [or its analogue] would trigger the initiation of transcription of lac genes [7].

Protein extraction and purification:

After 12 hours of induction the cells were harvested by centrifugation and washed in 100 ml per liter of original culture volume of buffer A [all reagents used in the purification was molecular biology grade, and care was taken to avoid contamination with biological material or metal ions]. Cells were recovered by centrifugation and resuspended in 50 ml per liter of original culture volume of pre-lysis buffer. After 15 minutes at room temperature an equal volume of lysis buffer were added and the lysis mixture was incubate at 75^0C for 1 hour. The lysis mixture then transferred to plastic bottles for centrifugation at 15,000 rpm for 10 minutes at 4^0C, and the clarified lysate then transferred to a clean flask. Taq polymerase was recover from the clarified lysate by adding 3.0 g of powdered $[NH_4]_2SO_4$ per 100 ml of lysate while stirring rapidly at room temperature. The solution were centrifuged at 15000 rpm for 10 minutes and then protein precipitated was harvested [both in pellets and as surface precipitate] and resuspended in 20 ml of buffer A per 100 ml of original cleared lysate. The resuspended protein was dialyzed for at least 12 hours, with storage buffer at 4^0C. After dialysis the resulting protein was dilute at 1:1 with sterilized storage buffer and stored at -70^0C. The purified protein was analyzed by SDS PAGE [13].

RESULTS

Taq DNA polymerase is a very important enzyme for molecular biological studies such as DNA amplification and DNA sequencing by the PCR [14]. It is a standard product used in 90% of molecular biology labs today, scientists still must spend time optimizing their PCR reactions, which can be time-consuming and tedious. The culture of *T. aquaticus* and isolation and purification of Taq pol I is again a challenging [15]. Observance this prospect in mind the existing study were done to built-up a clone of Taq pol I in lab which was used for expression of gene and production of Taq pol I enzyme. In forthcoming the clone of Taq pol I can be used further for random mutagenesis and site directed mutagenesis which will create various variants of accessible Taq polymerase and screening to find out the best one which fits the necessities of molecular researchers.

PCR amplification of isolated Taq gene:

In agarose gel electrophoresis a precise 2.6 Kb fragment of Taq gene were obtain, which forecast with the purpose of desired Taq DNA fragment were amplified successfully. This finding is supported by several scientific groups [14, 16]. They worked on the PCR and gene-specific DNA primers designed by Engleke [17].

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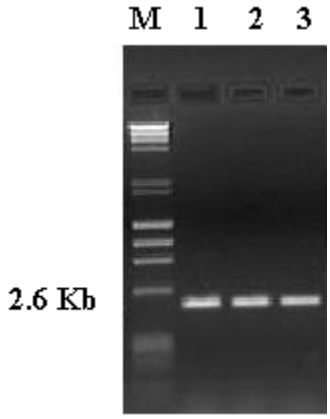

Figure-1: PCR amplification of *Taq* gene.

The Figure-1 depict that the band of 2.6 Kb consist desired amplified fragment of Taq DNA suggests the amplification of Taq DNA was done successfully. The 10 Kb ladder were load in lane M at the same time as lane 1st, 2nd and 3rd contain the PCR amplified sample DNA. Amplification was tartan in 0.7% agarose gel electrophoresis. Band of 2.6 Kb [Taq DNA] was observed in lanes 1st, 2nd and 3rd.

Ligation of Taq gene into pET100 Directional TOPO® Vector:

The PCR amplified Taq gene was ligeted in pET100 Directional TOPO® Vector by mean of restriction enzymes EcoRI and SalI had used to cut the plasmid open. DNA ligase was used to ligate the DNA fragments into the pET100 Directional TOPO® Vector. Subsequent to the completion of ligation reaction the cloned vector was checked by means of 1.2% agarose gel electrophoresis shown in Figure-2, ligated pET100 Directional TOPO® Vector [5.7 Kb] together with restriction enzyme EcoRI and SalI was loaded in the lane 1st, furthermore 2nd and 3rd lane were loaded with ligated vector [8.2 Kb, vector plus inserted DNA] and unligated vector respectively. Whereas lane M laden with 10 Kb molecular marker. The study of lane 1st, 2nd and 3rd shows that the desired Taq gene [2.6 Kb] was successfully ligated into pET100 Directional TOPO® Vector [5.7 Kb].

Desai [18] have reported the cloning of Taq DNA polymerase into pUC18 plasmid. Other reports are also available regarding the cloning of this gene [9, 19, 20]. However, this study is the first to clone the Taq DNA polymerase gene using pET100 Directional TOPO® Vector. This is a more convenient and much faster procedure as compared to those used in other studies. Taq DNA polymerase gene has been successfully cloned. This would allow performing many studies including expression of this gene, mass production of the enzyme and introducing mutations for enhancing its performance.

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SDS PAGE analysis:

The expression of Taq gene induced by IPTG was established by means of SDS PAGE analysis of induced protein. In SDS PAGE analysis [Figure-3], band of near 94 KDa was obtained which conformed that Taq gene was expressed and fabricate desired Taq enzyme which is nearly homogeneous with molecular weight 94 KDa and few contaminating protein bands were also present. It is also found that the intensity of expressed Taq protein is lower than the Taq protein expressed by some group of molecular scientist [9, 11, 16].

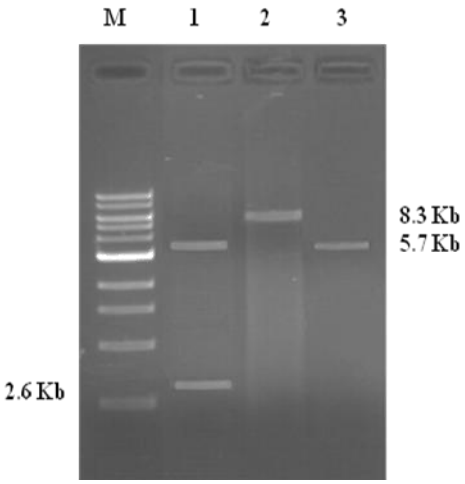

Figure 2: Ligation in pET100 Directional TOPO® Vector, lane 1st was loaded with ligated vector along with restriction enzyme *Eco*RI and *Sal*I, lane 2nd loaded with ligated vector, afterward lane 3rd were loaded with unligated vector. The lane M indicate molecular marker.

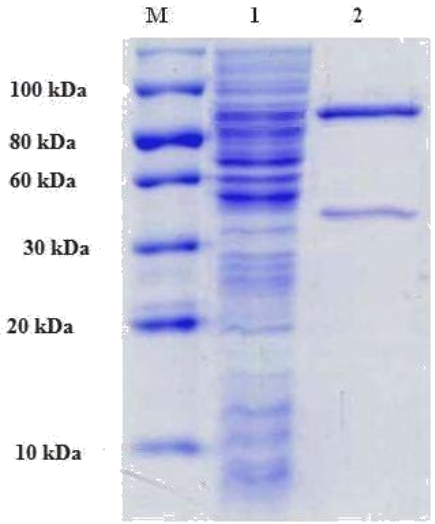

Figure 3: SDS-PAGE analysis of *Taq* DNA polymerase. Lane 1 was loaded with uninduced BL21 (DE3) supernatant, lane 2 were loaded with supernatant containing induced *Taq* pol I protein and lane M were loaded with 100 KDa marker.

CONCLUSION

The thermostable properties of the DNA polymerase activity from *T. aquaticus* have contributed greatly to the yield, specificity, automation, and utility of the PCR method for amplifying DNA. The Taq is ideal for both manual and automated DNA sequencing because it is fast, highly processive, has little or no 3'-exonuclease activity, and is active over a broad range of temperatures. The continuous demand of Taq in molecular lab increases the load in economics of the research laboratory. This economic load had generated an idea to develop Taq pol I in the labs at local level for the reduction of research cost, keeping this view in mind the present study were done to developed a clone of Taq pol I in lab which was used for expression of gene and production of Taq pol I enzyme.

A shortcoming in using Taq polymerase is its low fidelity rate compared to other types of polymerases. It is feasible, however, that a form of Taq with a higher fidelity rate could be developed. The challenge is fulfillment of rising demand of Taq DNA polymerase variants that add modified nucleotides for efficient DNA amplification and labeling, this challenge can be answered by using the techniques available in protein engineering like random mutagenesis and site directed mutagenesis which will create various variants of existing Taq polymerase and screening to find out the best one which fits the above necessities. A milestone would be achieved if we can develop optimized protocols for customizing protein of interest according to R&D/industrial needs, once we have an adequate quantity. The future of PCR based research lies in incorporating the desirable features of higher fidelity and thermostable DNA polymerases - Vent and Pfu, into Taq itself. This can therefore be achieved by altering the native gene of Taq Pol I to make mutant forms with improved expression in *E. coli*.

In this study the Taq gene was amplified from the genomic DNA of the organism *T. aquaticus* using PCR product was directly cloned into pET100 Directional TOPO® vector with use of restriction enzymes EcoRI and SalI. The *E. coli* BL21 was transformed using the recombinant pET100 [8.3 Kb] containing the gene of interest [Taq gene] and the clones of right orientation were selected and followed by protein induction. The IPTG was added for protein induction. Expression of gene was observed on SDS PAGE. The band observed around 94 KDa for Taq Pol I.

Because of the widespread use of Taq DNA polymerase, the main objective was to amplify, cloning and expression of its gene. Several attempts varying the experimental conditions, such as PCR cycles and $MgCl_2$ concentrations were made for its amplification without any success. Desai [18] have reported the cloning of Taq DNA polymerase into pUC18 plasmid. Other reports are also available regarding the cloning of this gene [9, 19]. The optimization of different molecular technique made possible to clone Taq DNA polymerase gene successfully in lab which allows the forthcoming student to perform many studies including expression of this gene, mass production of the enzyme and introducing mutations for enhancing its performance.

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