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CLONING AND CHARACTERIZATION OF ISOLATED TAQ DNA POLYMERASE GENE FROM PHAGE

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ABSTRACT
Taq polymerase is a thermo stable DNA polymerase named after the thermophilic bacterium *T.aquaticus,* having a molecular weight of 66,000-94,000 Daltons. Taq DNA polymerases have become a valuable tool in today's research. The choice of the DNA polymerase is determined by the goals of the experiment. We have isolated Taq DNA polymerase gene (2500 bp) from phage (PHI4-2) genomic DNA (USA) and cloned into a salt inducible bacterial expression vector (PUB-S-X-CH2) and the recombinant vector was transformed into BL21 (DE3) pLysS competent cells and screened for the presence of the insert in right orientation. The expression of Taq gene using salt inducible expression vector makes the production of Taq DNA polymerase enzyme much cheaper as compared to IPTG inducible expression systems.

INTRODUCTION

Molecular cloning refers to the procedure of isolating a defined DNA sequence and obtaining multiple copies of it *in vivo*. The process is frequently employed to amplify DNA fragments containing genes but it can also be used to amplify any DNA sequence such as promoters, non-coding sequences and randomly fragmented DNA. This can be utilized in a wide array of biological experiments such as large scale protein production. For efficient and direct molecular cloning (Mead *et al.,* 1991) the requirements are specific such as a host organism, a vector DNA, desired gene, amplification, selection, and screening techniques. DNA polymerases are key enzymes in the replication of genome containing all the cellular information that is present in all living things. Starting with the isolation and characterization of DNA polymerase I from *Escherichia coli* by Kornberg and colleagues in the 1950s (Kornberg, 1980), since then more than 50 DNA polymerases have been cloned and sequenced from various organisms, including thermopiles and archaea.

The thermo stable properties of the Taq DNA polymerase (Taq) from *Thermus aquaticus* have contributed greatly to the yield, specificity, automation, and utility of the polymerase chain reaction methods (Mullis, 1986) for amplification of DNA has become a valuable tool in today's molecular research. The application of thermostable DNA polymerase in PCR makes the automation of PCR possible. *Taq* DNA polymerase from *Thermus aquaticus* was the first characterized thermostable enzyme (Chien, 1976). After words, many DNA polymerases from the *Thermus* strain were studied and *Tfi, Top, Tfl,* and *Tth* polymerase have been applied to PCR (Kaledin *et al.*, 1981; Ruttimann *et al.*, 1985; Jung *et al.*, 1997; Kim *et al.*, 1998); but, their base-insertion fidelity was somewhat low since these DNA polymerases do not have 3'→5' exonuclease activity in them. The high-fidelity of DNA polymerases, which has 3'→5'exonuclease-dependent proofreading activity, are required for error correction during the polymerization. Many thermostable DNA polymerases with proofreading activity (*Vent, deep Vent, Pfu,* and *Pwo*) have also been studied and introduced for high-fidelity PCR amplification (Cariello *et al.*, 1991; Cline *et al.*, 1996). Many thermostable enzymes were synthesized at very low levels by the thermophilic bacteria, therefore, they were difficult to purify. Then thermostable DNA polymerase, such as *Pfu* DNA polymerase, was produced in biologically active form in the *E.coli* over-expression system (Lu and Erickson, 1997; Dabrowski and Kur, 1998). However, several problems persist, such as error-prone amplification and unwanted amplification at low temperatures in the PCR applications. Taq DNA polymerase has a terminal transferase activity and it adds an A (adenine) at 3' end (Magnuson *et al.*, 1996) which was found to be quite useful and was exploited to produce TA cloning vector (plasmid) which possess a 3'-T (Thymine) overhangs. This allows ligation using DNA ligase to quickly be accomplished with the A overhangs of the PCR product. In our experiment we used LE392 bacterial (ATCC, USA) strain

that lacks *E.coli* K restriction system and allows the growth of PHI4-2 phage. The isolated genomic DNA of PHI4-2 phage was partially digested with the help of specific restriction endonuclease and the desired gene for Taq DNA polymerase was eluted after its electrophoresis (Sambrook *et al.*, 1989). The eluted and purified gene was cloned within pUB-S-X-CH2 plasmid vector (isolated from JM109, promega bacterium). The cloned recombinant DNA was allowed to transform into BL21 (DE3) pLysS bacterium (derivative of *E. coli* and deficient in both lon and ompT proteases) and screened for the positive recombinants by colony PCR method.

MATERIALS AND METHODS

Isolation of Phi 4-2 Genomic DNA

Phage PHI4-2 genomic DNA containing Taq polymerase gene was isolated by inoculating LE392 bacterial (ATCC, USA) strain in lambda media (Himedia) containing phage lysates (ATCC, USA) diluted to 1:10 in lambda diluents which were diluted in LB media (Himedia) and after incubation a single plaque from a plate containing no more than 100 plaques (Fig. 1) was picked up and transferred to fresh LE392 culture and incubated for 18h under constant shaking and at 37°C. After incubation clear lysate was treated to purify the phage DNA using SDS, proteinase K and desalting methods. The experimental setup included LE392 (negative control) and phage infected LE392 lysate. Phage genomic DNA was eluted in elution buffer and was analyzed on 1% agarose gel electrophoresis and compared with 1kb ladder (Fig. 2). Single band indicated the good quality of isolated DNA and lack of DNA band from negative control proved that the single band DNA was of phage.

Isolation of Taq Gene Dna Fragment From Phi4-2 Genomic Dna

The isolated PHI4-2 genomic DNA was undergone specific restriction endonuclease digestion (EcoRI) for the isolation of Taq gene containing DNA fragment. The DNA fragments were separated by agarose gel electrophoresis and the desired band was excised from the gel with the help of scalpel and placed in the micro-centrifuge vial along with DNA binding buffer and the desired DNA fragments were eluted with the elution buffer and stored at -20°C. After that quantification of the isolated DNA fragment was done photometrically.

Isolation of Plasmid Vector Pub-S-X-Ch2 DNA

Plasmid DNA was isolated by seeding a single colony of bacteria (JM109, promega, genotype: endA1,recA1, gyrA96, thi, hsdR17 rk^- and mk^+ , relA1, supE44, Ä lac-proAB, F′ traD36, proAB, laqIqZÄM15) in 10ml of LB media (Himedia, autoclaved at 121°C for 20 min at 15psi) containing 100 μg/mL of ampicillin (Himedia), incubated in an orbital shaker (REMI) at 37°C, 200 rpm for 18 hr, after which culture was centrifuged at 13000 rpm for 30 sec followed by desalting and extracting the plasmid which was stored at -20°C.

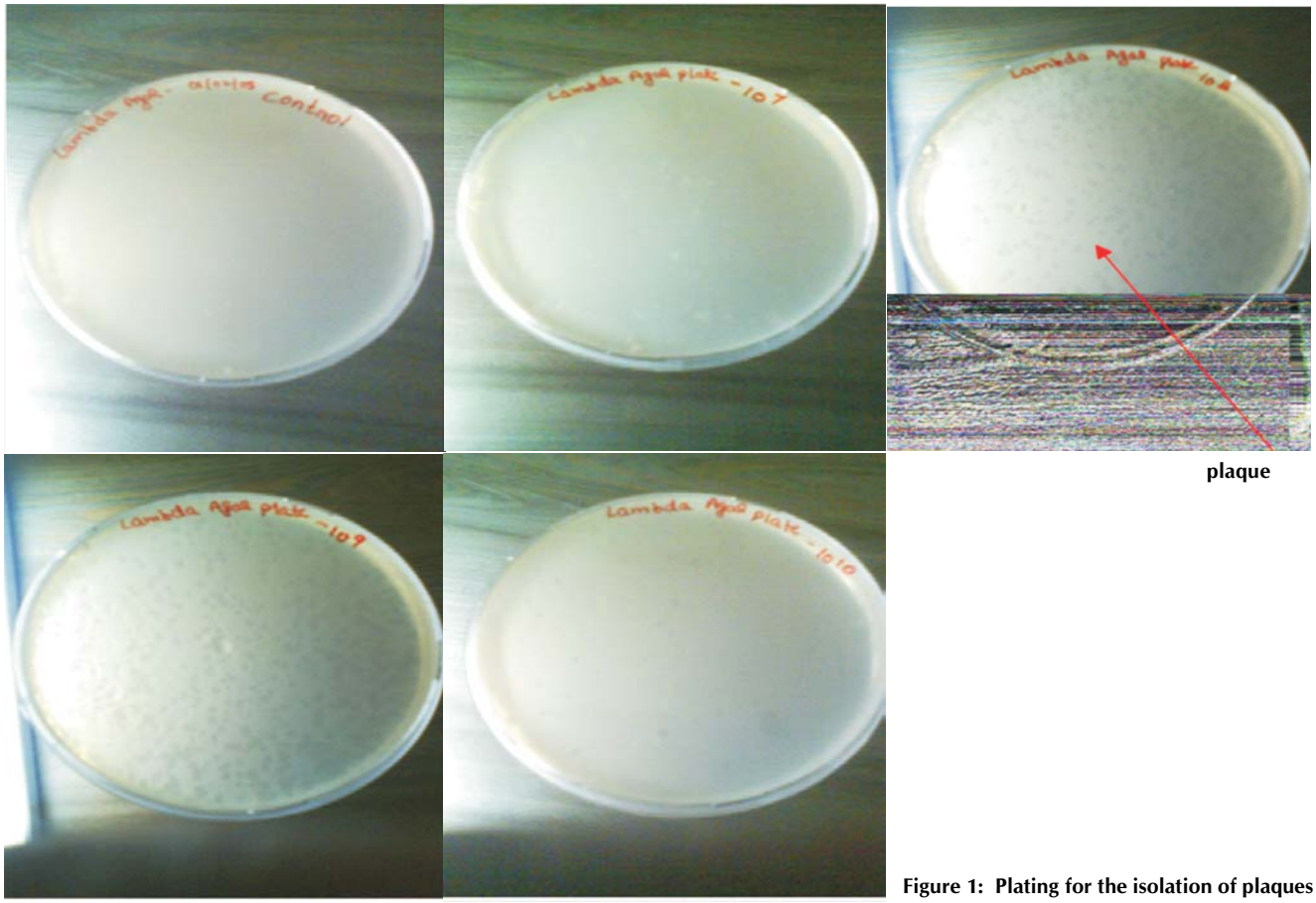

Figure 1: Plating for the isolation of plaques

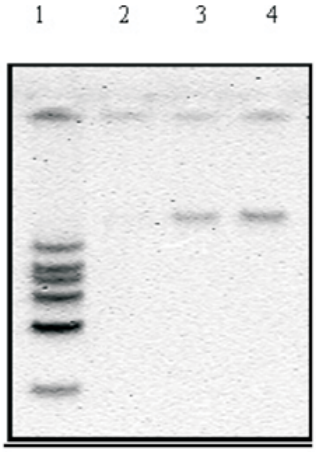

Figure 2: Isolation of Phage Genomic DNA: Lane 1 (1 kb ladder), Lane 2 (Phage genomic DNA prep of LE392, -ve control) and Lane 3 and 4 (Phage genomic DNA prep of phage infected LE392

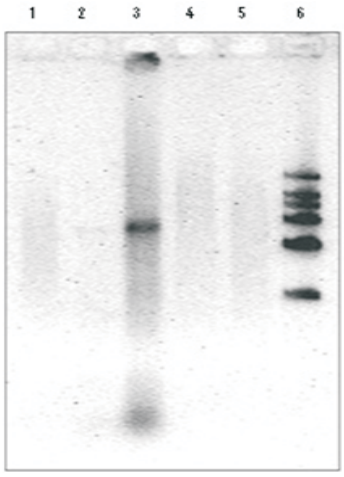

Figure 3: Amplification of Taq DNA polymerase gene with different concentrations of $MgCl_2$: Lane 1 (2mM $MgCl_2$), Lane 2 (3mM $MgCl_2$), Lane 3 (4mM $MgCl_2$), Lane 4 (5mM $MgCl_2$) and Lane 5 (-ve control) and Lane 6 (1kb ladder)

Pcr Amplification of Taq Gene

Taq DNA polymerase DNA fragment from phage DNA was amplified using Taq-F and Taq- R primers (Table 1) in the presence of 25mM $MgCl_2$ (AR, Loba Chem.). The setup for PCR reaction (Harris Jones, 1997) and the cycling conditions (Table 2 and 3) were adjusted and following amplification the samples were subjected to electrophoresis (Fig. 3) from which a 2500 bp of amplified Taq DNA was eluted in elution buffer.

Cloning of Taq Gene In Pub-S-X-Ch2 Plasmid

Pub-S-X-CH2 is a salt inducible expression and TA cloning vector which facilitates direct cloning of PCR amplified genes without the use of restriction enzymes (Holton and Graham, 1990). It also consists of His Tag coding sequence downstream to the multiple cloning site to facilitate the isolation of Taq by affinity chromatography. Taq DNA polymerase gene (2500 bp) fragment was ligated (Vigneault. *et al.*, 2005) with pUB-S-X-CH2 linear vector, a backbone control was also included to distinguish the presence of backbone regulation (Table 4). Ligation was carried out using T4-DNA ligase at 4°C for 24h before transformation.

Table 1: Oligos for the amplification of taq dna polymerase gene

O. P. name	Olig Sequence
Taq-F	AGGAAAGAGCTATGAGGGGGATGCTGCCCCTCTTT
Taq-R	TTACTCCTTGGCGGAGGAGAGCCAGTC
Scr-O (Reverse Primer) For Colony PCR	CCTACTCAGGAGAGCGTTCA

Primers were designed on Vector NTI Software; O. P. Name = Oligo primer name

Table 2: Pcr program

Step 1	94°C	2min
Step 2	94°C	1min
Step 3	55°C	1min
Step 4	72°C	1min
Step 5	Repeated Step- 2 to 4 (30 cycles)	
Step 6	72°C	10min
Step 7	Hold at 4°C	

Transformation of Cloned Recombinant Dna In Bl21 (De3) pLysS

Cloned recombinant DNA were transformed into BL21 (DE3) pLysS $CaCl_2$ (AR, Loba Chem.) competent cells by heat shock method for 90 sec. followed by ice incubation for 2 min. LB media was added to the cells and incubated at 37°C for 40 min after which they are plated on LB/Amp plate.

Screening of Clones for The Orientation of Taq Gene In Pub S-X-Ch2

Clones were screened for the right orientation using colony PCR method (Table 5) in which three clones from pUB S-X-CH2$^+$ Taq plate were used with Taq-F primer and Scr-O Reverse primer (Table 1). Scr-O reverse primer annealed downstream to the His-Tag on the vector. One clone gave a fragment of size of 2500 bp indicating the right orientation of cloned DNA in the vector (Fig 4).

RESULTS AND DISCUSSION

Taq DNA polymerase (2500 bp fragment) was isolated from the phage genomic DNA and was cloned into the salt inducible bacterial expression vector (pUB S-X-CH2). The recombinant Taq expression vectors make the production of Taq much cheaper when compared to IPTG inducible expression systems. Further studies on the isolation of Taq DNA polymerase have to be done on the BL21-Taq clone generated in here for it to be used for the production of Taq DNA

Table 3: Amplification of taq dna polymerase: reaction setup

Components	Tube 1	Tube2	Tube 3	Tube 4	Tube 5
10 x PCR buffer	2μL	2 μL	2 μL	2 μL	2 μL
$MgCl_2$ (25mM)	1.6 μL	2.4 μL	3.2 μL	4 μL	1.6 μL
dNTP (10mM)	0.8 μL	0.8 μL	0.8 μL	0.8 μL	0.8 μL
Taq (3μg/μL)	0.5 μL	0.5 μL	0.5 μL	0.5 μL	0.5 μL
Phage genomic DNA	1 μL	1 μL	1 μL	1 μL	-
H_2O	13.9 μL	13.1 μL	12.3 μL	11.5 μL	14.9 μL

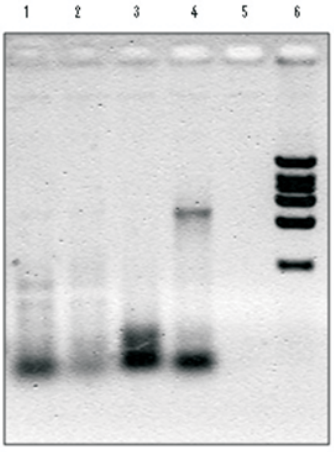

Figure 4: Screening of pUB-S-Taq-CH2 vectors for the orientation of Taq gene; Lane 1 (Clone 1), Lane 2 (clone 2), Lane 3 (clone 3), Lane 4 (clone 4), Lane 5 (-ve) and Lane 6 (1kb ladder)

Table 5: Colony PCR reaction setup

Components	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5
10 x PCR buffer	2 μL	2 μL	2 μL	2 μL	2 μL
$MgCl_2$ (25mM)	1.6 μL	1.6 μL	1.6 μL	1.6 μL	1.6 μL
dNTP (10mM)	0.8 μL	0.8 μL	0.8 μL	0.8 μL	0.8 μL
Taq (3 μg / μl)	0.5 μL	0.5 μL	0.5 μL	0.5 μL	0.5 μL
Phage genomic DNA	1 μL	1 μL	1 μL	1 μL	-
H_2O	12.1 μL	12.1 μL	12.1 μL	12.1 μL	13.1 μL

protein. Positive clones were tested for the orientation of gene which is very important for the expression of recombinant genes. pUB S-X-CH2 generated in the present study may be used in the expression of Taq DNA polymerase in presence of NaCl, making it an economical and regulated expression vector for the production of Taq.

Table 4: Cloning of Taq DNA polymerase gene in pUB-S-X-CH2 plasmid

	pUB-S-X-CH_2	PCR amplified Taq	T4 Ligase Buffer	T4 DNA Ligase	H_2O
1 (back bone control)	2μL	-	1 μL	1 μL	6 μL
2	2 μL	6 μL	1 μL	1 μL	-

polymerase in BL21. Several groups have reported the cloning and expression of genes from thermophiles in *E. coli* (Lawyer *et al.*, 1989). Many others have also reported the cloning and expression of DNA polymerase in *E.coli*. and cloned the structural gene for DNA polymerase (Pol-I) from *E.coli. in ë* bacteriophage (Lleonart *et al.*, 1992). They observed polymerase activity in the transducing phage at a level of approximately 4% of total cell protein. However they are unable to maintain a plasmid harboring the Pol A^+ gene, probably because overproduction of Pol I in *E. coli* was lethal to the cell. Some gene products are toxic to the bacterial cells. We don't know the detrimental activity of Taq DNA polymerase on the *E. coli* cells, so we preferred to clone Taq under salt inducible promoter for better control over the expression of recombinant Taq.

PHI4-2 (ATCC) encodes for the complete Taq DNA polymerase gene. PHI4-2 was plated along with the LE392 to isolate a single plaque. From our experiment pfu of PHI4-2 was found to be $29X10^{11}$pfu/mL. Plaques formed on the plate were then used to infect LE392 and isolated the genomic DNA of the phage containing the desired gene of Taq DNA polymerase enzyme. Comparative studies on phage infected and uninfected LE392 cells revealed that the method used in the present investigation was found efficient and better in comparison to other methods using toxicant chemicals. Taq-F and Taq-R primers were designed from the sequence available from Lawyer *et al.,* 1989. The PCR amplification of Taq DNA polymerase using Taq-F and Taq-R primers was found to be very specific. Taq was amplified in presence of 25mM $MgCl_2$ (AR, Loba Chem.). (Saiki *et al.*, 1988). PCR amplified Taq DNA polymerase gene was cloned into pUB S-X-CH2 plasmid. The plasmid was derived from pOSEX-2 which consists of osmotic regulated promoter (ProU), which facilitates the expression of Taq in the presence of high concentration of NaCl. It is a TA cloning vector that facilitates direct cloning of Taq DNA polymerase amplified PCR products which add an A at its 3' end (Crowe *et al.*, 1991). The plasmid also encodes for His-Tag (Lin *et al.*, 2007) which helps in the isolation of

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