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Cost-effective, high-yield production of *Pyrobaculum calidifontis* DNA polymerase for PCR application

Kashif Maseh[a], Syed Farhat Ali[a], Shazeel Ahmad[b], and Naeem Rashid[b]

[a]KAM School of Life Sciences, Forman Christian College (A Chartered University), Lahore, Pakistan; [b]School of Biological Sciences, University of the Punjab, Lahore, Pakistan

ABSTRACT

Polymerase Chain Reaction (PCR) is widely used for cloning, genetic engineering, mutagenesis, detection and diagnosis. A thermostable DNA polymerase is required for PCR. Here we describe low-cost and high-recovery production of *Pyrobaculum calidifontis* DNA polymerase (*Pca*-Pol). The gene was cloned in pET-28a and expressed in *Escherichia coli* BL21CodonPlus. Gene expression conditions were optimized. Eventually, gene expression was induced with 0.1 mM IPTG for 3 hours at 37 °C. Recombinant *Pca*-Pol produced was purified to homogeneity by immobilized metal-ion affinity chromatography yielding around 9000 U of *Pca*-Pol per liter of the culture with a recovery of 92%. Stability and PCR amplification efficiency of *Pca*-Pol was tested under various storage conditions with highest efficiency in 25 mM Tris-Cl buffer (pH 8.5) containing 0.1% Tween 20, 0.2 mg/mL BSA and 20% glycerol. Under this condition, no loss in PCR activity of *Pca*-Pol was observed, even after one year of storage. Repeated freeze-thaw, however, deteriorated enzyme activity of *Pca*-Pol. 55% PCR amplification activity retained after 7 prolong freeze-thaw cycles (freezing overnight at −20 °C and thawing for 45 minutes at 28 °C). Purified *Pca*-Pol possessed 3′–5′ exonuclease (proofreading) activity and is expected to have greater fidelity as compared to *Taq* polymerase which does not have proofreading activity.

KEYWORDS

DNA polymerase; expression optimization; polymerase chain reaction (PCR); purification; *Pyrobaculum calidifontis*; stability

Introduction

Archaea constitute the third domain of life. Over past years, a lot of new species of this domain have been discovered and characterized - many of which are extremophiles.[1] Initially Euryarchaeota and Crenarchaeota were the two main phyla of this domain. However, with broader environmental sampling, many new archaeal species were discovered which do not fall in these phyla. This led to a redesign of archaeal taxonomy. Currently the archaeal superphyla include Asgard, DPANN and TACK.[2] Archaea have gained much attention because of extreme environments they dwell. Their enzymes are stable in harsh environments and find application in a variety of industrial processes.[3] Some examples include carbohydrate acting enzymes, proteases, lipases, dehydrogenases, isomerases and DNA polymerases.[4,5]

Polymerase chain reaction (PCR) is a valuable technique used in genetic engineering, diagnostics and forensics. Over time, many technological advancements have been made in this field.[6] Various PCR-based methods are used for diagnosis of diseases and infections.[7–9] PCR involves heating at high temperatures, so a thermostable DNA polymerase is required for this process.[4] Hyperthermophilic archaea are particularly important in this regard, as their DNA polymerases are stable at high temperatures.[5] Archaea are known to have DNA polymerases belonging to B-family (PolB) and D-family (PolD).[10] Based on sequence and phylogenetic analyses, archaeal PolBs have been characterized into various groups, some of which have close relationship with their eukaryotic counterparts.[11] Various B-family archaeal DNA polymerases have been characterized and some are commercially available as well.[12]

Pyrobaculum calidifontis (*Pca*) is a hyperthermophilic archaeon that grows optimally at 90–95 °C and pH 7.[13] The B-family DNA polymerase from *P. calidifontis* (*Pca*-Pol) is a magnesium-dependent thermostable enzyme with a half-life of 4.5 hours at 95 °C and can amplify up to 7.5 kb DNA in PCR.[14] Here, we describe the expression of His-tagged *Pca*-Pol for affinity purification. Optimization of gene expression and affinity purification have resulted in cost-effective production of *Pca*-Pol with high recovery while retaining polymerase and proofreading activities. The stability of *Pca*-Pol during storage and effect of freeze-thaw on PCR performance are also reported.

Materials and methods

Strains, chemicals and media

Plasmid pET28a and host strain *E. coli* BL21CodonPlus (DE3) RIL were obtained from Novagen (USA). All enzymes, deoxyribonucleoside triphosphates (dNTPs), GeneJet plasmid

CONTACT Syed Farhat Ali farhatali@fccollege.edu.pk KAM School of Life Sciences, Forman Christian College (A Chartered University), Lahore, Pakistan.
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isolation kit, DNA and proteins markers were purchased from Fermentas (Canada). TTP [methyl- ^{3}H] was from MP Biomedicals (USA). Activated calf thymus DNA was from Sigma (USA). Other chemicals, reagents and growth media components were from either Merk or Sigma.

Cloning and transformation

Pca-pol gene previously cloned in pTZ57R/T[14] was digested with *Nde*I and *Bam*HI, gel purified and ligated to pET28a digested with the same restriction endonucleases. The resulting recombinant expression vector was named *Pca-pol*-pET28a. The map of resulting plasmid is shown in Supplementary Figure S1. *E. coli* BL21 CodonPlus (DE3) RIL cells were transformed with plasmid *Pca-pol*-pET28a and plated on LB agar containing 50 µg/mL kanamycin.

Optimization of gene expression

A colony of *E. coli* BL21 CodonPlus (DE3) RIL cells, transformed with recombinant plasmid *Pca-pol*-pET28a, was inoculated in 20 mL LB broth (containing 50 µg/mL kanamycin) and incubated overnight at 37 °C with shaking at 200 rpm in an orbital shaker. 1% of the overnight grown culture was used to initiate 50 mL secondary culture for optimization of gene expression. IPTG concentration was optimized in a range of 0.1–1 mM. To analyze the effect of growth phase on gene expression, induction was done at culture OD_{600} of 0.2, 0.4, 0.6, and 0.8 and an uninduced culture was used as control. Post-induction time was optimized by taking a culture sample at one hour interval (after IPTG addition) up to five hours. Effect of growth temperature on gene expression was also studied by carrying out gene expression at 20, 30, and 37 °C. All experiments were done in triplicates.

SDS-PAGE and densitometric analyses

Collected culture samples were subjected to 12% SDS-PAGE. Gel images were captured by using Chemi Doc™ XRS + System (Bio-Rad) and analyzed with Image Lab™ Software (Bio-Rad). Known amount of BSA was used as a reference for determination of protein concentration form gel. Densitometry analysis was done by normalizing gel-loading and subtracting the background i.e., uninduced control. Protein concentration of samples was measured by using Bradford method.[15]

Expression up-scaling and purification of Pca-Pol

After optimization, gene expression was done in 500 mL culture in LB broth containing 50 µg/mL kanamycin with shaking at 200 rpm at 37 °C. IPTG (0.1 mM) was added at OD_{600} of 0.6 and grown for 3 hours. After which cells were harvested by centrifugation at 6500 x g for 15 min at 4 °C. The cell pellet was suspended in 25 mM Tris-Cl pH 8.5 and lyzed by sonication using Sonics Vibra Cell. Sonication was done at an amplitude of 40% with 20 seconds pulse on and 10 seconds off. Clear lysate was collected by centrifugation at 12000 x g for 15 min at 4 °C. The supernatant obtained was heated at 80 °C for 20 minutes to denature host-cell proteins. After which it was centrifuged again to get heat-stable supernatant. Ni-NTA resin was added to heat-stable supernatant and kept on a platform mixer at 4 °C for protein binding. The resin (with bound *Pca*-Pol) was collected by centrifugation at 1200 × g at 4 °C for 2 minutes and washed with 25 mM Tris-Cl pH 8.5 containing 10 mM imidazole. After washing, *Pca*-Pol was eluted by using step gradient of 50 mM − 1 M imidazole in 25 mM Tris-Cl pH 8.5. The fractions obtained were analyzed by 12% SDS-PAGE and enzyme assay. Protein concentration of obtained fractions was determined by using Bradford method.[15]

Analysis of production cost of Pca-Pol

Cost of production of His-tagged *Pca*-Pol was determined by adding the cost of the following: chemicals and media used for gene expression under optimized conditions; chemicals and reagents used for affinity purification; electricity cost during expression and purification. This was compared to cost of production of un-tagged *Pca*-Pol. All costs were determined in Pakistani Rupees (PKR) and converted to US dollars (USD).

Assay for DNA polymerase activity

Assay for DNA polymerase activity was done as describe previously[14] by measuring the incorporation of TTP [methyl-^{3}H] by using activated calf thymus DNA. The reaction mixture, in 20 µL, contained: 25 mM Tris–Cl pH 8.5, 4 mM $MgCl_2$, 100 µM each of dATP, dGTP, dCTP, dTTP, 0.5 µCi TTP [methyl-^{3}H] (78 Ci/mmol), 5 µg activated calf thymus DNA, 0.2 mg/ml BSA and 0.1% Tween 20. The reaction mixture was incubated at 75 °C for 5 min before enzyme addition. After adding the enzyme, aliquots were removed from the reaction mixture at various time intervals and spotted onto DE-81 filter paper disks (23 mm diameter, Whatman). The disks were air-dried, washed three times in sodium phosphate buffer pH 7.0 and finally washed with 70% ethanol. All washing steps were done for 2 min each. The filter paper disks were then dried and incorporated radioactivity on the filter disks was measured in counts per second (cps) by using Raytest Malisa scintillation counter (Berlin, Germany). One unit of DNA polymerase activity was defined as the amount of the enzyme required to incorporate 10 nmol [methyl ^{3}H] TTP into a polynucleotide fraction (adsorbed on DE-81 filter disc) at 75 °C in 30 min.

Analysis of exonuclease activity

The 3′→5′ exonuclease activity of *Pca*-Pol was analyzed by monitoring the degradation of a 40-mer primer (5′-biotin-CGCACCGTGACGCCAAGCTTGCATTCCTACAGGTCGACTC-3′) annealed to an 80-mer template (5′-biotin-CGTTGCTGACAAACGGGCCGGTCAACAATCCTCTGGAGTCGACCTGTAGGAATGCAAGCTTGGCGTCACGGTGCGCCAAC-3′).[14] To the annealed primer-template was added

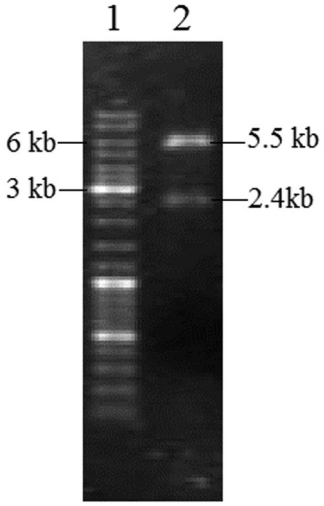

Figure 1. Double digestion of isolated recombinant plasmid *Pca-pol*-pET28a. Lane 1: DNA ladder; Lane 2: *Pca-pol*-pET28a digested with *Nde*I and *Bam*HI to release *Pca-pol* gene (2.4 kb).

1 U of Pca-Pol and the mixture was incubated at 75 °C for 1 h. After incubation, DNA was resolved on 15% polyacrylamide gel containing 7 M urea, transferred to Hybond N + membrane (GE Healthcare) using Invitrogen XCell Surelock Blot Module and detected by using biotin chromogenic detection kit (Fermentas).

PCR with Pca-Pol

PCR performance of *Pca*-Pol was monitored in *Pca*-Pol buffer which contained 25 mM Tris-Cl buffer pH 8.5 containing 0.1% Tween 20 and 0.2 mg/mL bovine serum albumin (BSA). A 2.6 kb DNA fragment cloned in a plasmid was used as template. The reaction mixture, in 20 µL of *Pca*-Pol buffer, contained 1 ng template plasmid, 1 µM each forward and reverse primer, 4 mM $MgCl_2$, 200 µM dNTPs and 1 U *Pca*-Pol. After PCR, the reaction mixture was subjected to agarose gel electrophoresis. PCR amplification of *Pca*-Pol was also compared to that of commercially available *Pfu* and *Taq* DNA polymerases according to the method described by the manufacturer.

Analysis of storage conditions and effect of repeated freeze-thaw

Purified *Pca*-Pol was stored at −20 °C in 25 mM Tris-Cl buffer pH 8.5 containing glycerol (20 or 50%) with or without 0.2 mg/mL BSA and 0.1% Tween 20. After 2 months of storage, PCR was carried out with 1 U of enzyme stored in various buffers compositions to determine the optimal storage buffer retaining maximum enzyme activity. *Pca*-Pol was then stored in optimal buffer at −20 °C for one year and its PCR amplification efficiency was compared to freshly produced enzyme.

Effect of prolong thawing and freezing on PCR performance of the enzyme was also evaluated. A fresh batch of *Pca*-Pol was stored in optimal storage buffer at −20 °C. The enzyme was subjected to cycles of freezing overnight at −20 °C and thawing for 45 minutes at room temperature (28 °C). After repeated freeze-thaw cycles, residual enzyme activity was evaluated by its PCR amplification efficiency.

Results

Gene cloning

Previously cloned *Pca-pol* gene in pTZ57R/T was digested and subsequently cloned in pET28a between *Nde*I and *Bam*HI sites. After transformation of *E. coli* BL21 CodonPlus (DE3) RIL with *Pca-pol*-pET28a, the recombinant plasmid was isolated and double digested with *Nde*I and *Bam*HI. Double digestion released the *Pca-pol* gene (∼2.4 kb) from the vector pET28a (∼5.5 kb) as shown in Figure 1.

Optimization of gene expression

Following factors were optimized for gene expression: pre-induction growth phase, post-induction growth time, growth temperature and IPTG concentration. For optimization of inducer concentration, IPTG was added (final concentration 0, 0.05, 0.1, 0.2, 0.5, and 1 mM) at culture OD_{600} of 0.6. After growth of 4 h, cells were harvested and analyzed for gene expression by SDS-PAGE followed by densitometric analysis. Maximum yield of *Pca*-Pol was observed when gene expression was carried out with 0.1 mM IPTG. At this inducer concentration, *Pca*-Pol constituted approximately 30% of total cell proteins as shown in Figure 2a. No significant increase in expression was observed by further increasing IPTG concentration. So, IPTG concentration of 0.1 mM was selected for further use.

Pre-induction growth phase and post-induction growth time were optimized simultaneously. Cultures were induced with 0.1 mM IPTG at OD_{600} of 0.2, 0.4, 0.6, and 0.8 and samples were collected between 1 to 6 hours of IPTG addition from each culture. The result obtained is shown in Figure 2b. Highest expression of *Pca*-Pol was obtained when induction was done at OD_{600} of 0.6 for 3 hours. Under the tested conditions, expression increased with time up to 3 hours and later decreased gradually. After 6 hours of post-induction incubation, expression decreased to almost half of maximum.

So, gene expression was done by adding 0.1 mM IPTG at OD_{600} of 0.6. Accumulation of cell-mass was monitored by taking culture density at each interval of time. Although cell mass kept on increasing throughout the duration studied, but the amount of *Pca*-Pol produced started to decrease significantly after 4 hours and reduced to almost half after 6 hours as shown in Figure 2c.

To evaluate the effect of temperature on the production of *Pca*-Pol, cultures were incubated at three different temperatures (20, 30, and 37 °C). Other conditions were kept constant as already optimized. Culture samples were analyzed by SDS-PAGE. Expression was observed at all temperatures. However, maximum expression occurred at 37 °C as shown in Figure 2d. Therefore, for initiating large-scale

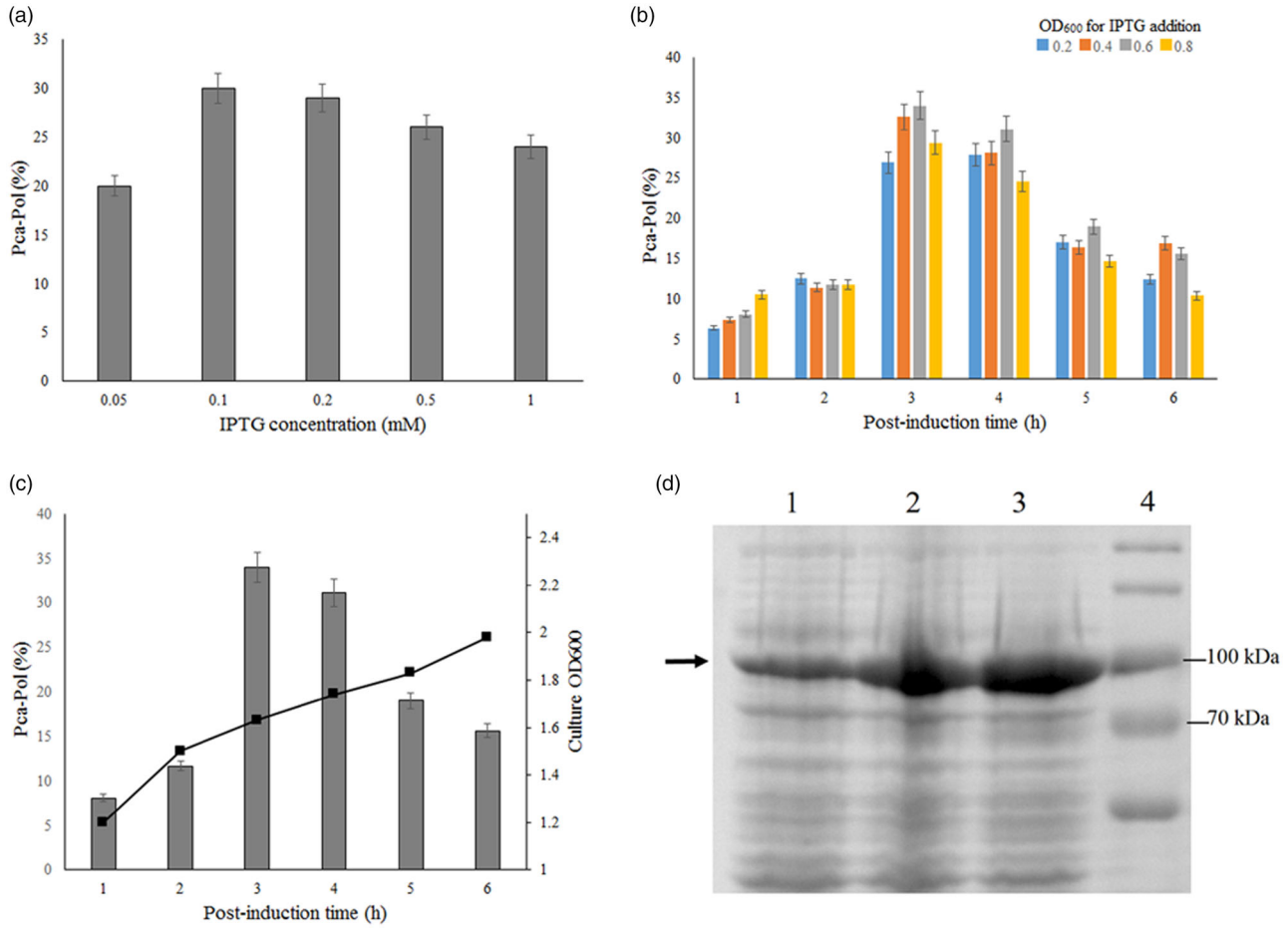

Figure 2. (a) Optimization of IPTG concentration for *Pca-pol* gene expression. IPTG concentration (mM) is represented on x-axis. *Pca*-Pol as a percentage of total cell protein is represented on y-axis. (b) Optimization of pre- and post-induction growth time. Gene expression was induced at various culture densities. Each bar show OD_{600} at which IPTG was added. Post-induction time (h) for each treatment is shown on x-axis. *Pca*-Pol as a percentage of total cell protein is represented on y-axis. (c) Relationship between *Pca*-Pol production and cell biomass accumulation. Post-induction time (h) is represented on x-axis and *Pca*-Pol as a percentage of total cell protein is represented on y-axis. Secondary y-axis (right) represents culture OD_{600} at each interval of time. (d) Effect of growth temperature on the production of *Pca*-Pol. Lane 1: 20 °C; Lane 2: 30 °C; Lane 3: 37 °C; Lane 4: Protein ladder. The position of *Pca*-Pol is marked by an arrow.

culture, gene expression was induced with 0.1 mM IPTG at OD_{600} of 0.6 for 3 hours at 37 °C.

Purification of Pca-Pol

Gene expression of *Pca*-Pol was done in 500 mL cultures under optimized conditions. After which, cells were harvested, sonicated and the resulting lysate was heated at 80 °C for 20 minutes to denature host proteins. *Pca*-Pol was found in soluble fraction after heating and purified by affinity chromatography (IMAC). As shown in Figure 3, *Pca*-Pol was purified to near homogeneity after single-step affinity purification. The fractions obtained were analyzed by enzyme assay. Approximately 18 mg (~9000 U) of purified *Pca*-Pol was obtained per liter of the culture, with a recovery of 92%. The purification process is summarized in Table 1.

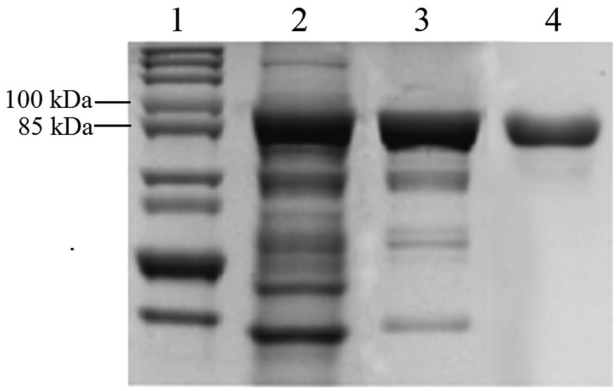

Figure 3. SDS-PAGE analysis of *Pca*-Pol purification. Lane 1: protein ladder; Lane 2: cell lysate after gene expression; Lane 3: heat-stable supernatant, Lane 4: *Pca*-Pol after affinity purification (IMAC).

Cost-analysis for Pca-Pol production

His-tagged *Pca*-Pol was produced under optimized expression conditions. The cost of production was calculated as described in methods. It was found that under optimized conditions, the production cost of His-tagged *Pca*-Pol was significantly lower than that of untagged *Pca*-Pol. Expression optimization and affinity purification resulted in reducing the production cost by 87%. This was primarily because of high recovery (92%) of His-tagged *Pca*-Pol by affinity purification after gene expression under optimized conditions. Almost 9000 U of purified Pca-Pol were obtained per liter of the culture. The cost of production was around USD 20.

Table 1. Summary of purification process of His-tagged *Pca*-Pol.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Fold purification	Recovery (%)
Crude extract	141.6	9710	68.5	1	100
Heat denaturation	56.7	9286	163.7	2.3	95.6
Affinity purification (IMAC)	17.5	8962	512.1	7.4	92.3

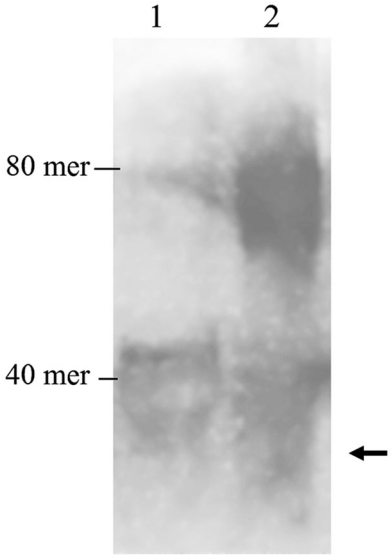

Figure 4. 3′–5′ Exonuclease activity of purified *Pca*-Pol. Lane 1: 40-mer only; Lane 2: 40-mer annealed to 80-mer in the presence of *Pca*-Pol. The arrow marks the degradation of 40-mer by *Pca*-Pol 3′–5′ exonuclease activity.

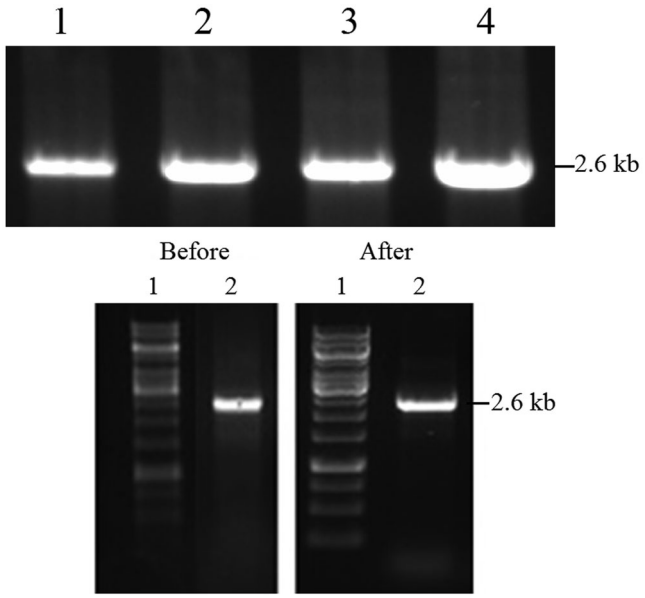

Figure 5. (a) Effect of storage buffer composition on PCR amplification of *Pca*-Pol. The storage buffer in all cases contained 25 mM Tris-Cl pH 8.5 along with the indicated components. Lane 1: 50% glycerol; Lane 2: 50% glycerol, 0.1% Tween 20, 0.2 mg/mL BSA; Lane 3: 20% glycerol; Lane 4: 20% glycerol, 0.1% Tween 20, 0.2 mg/mL BSA. (b). Analysis of *Pca*-Pol stability during storage in optimal buffer. The panel "Before" shows PCR amplification before storage and the panel "After" shows PCR amplification after 1 year of storage in optimal buffer at -20 °C. Lane 1 (both panels): DNA ladder; Lane 2 (both panels): 2.6 kb amplified product.

Exonuclease activity

3′-5′ Exonuclease activity confers proofreading ability to DNA polymerase. 3′-5′ exonuclease activity of *Pca*-Pol was analyzed by degradation of a 5′ biotin labeled 40-mer 'primer' annealed to an 80-mer template. *Pca*-Pol showed 3′-5′ exonuclease activity by degrading the primer (Figure 4).

Analysis of storage conditions for Pca-Pol

25 mM Tris-Cl pH 8.5 with various concentrations of additives was used to store *Pca*-Pol at −20 °C. Stored *Pca*-Pol was then used in PCR. It was found that *Pca*-Pol showed maximum activity in the following buffer: 25 mM Tris-Cl pH 8.5 containing 0.1% Tween 20, 0.2 mg/mL BSA and 20% glycerol with a storage temperature of −20 °C as represented in Figure 5a. The enzyme was then stored in this optimized buffer at −20 °C for a year. PCR was carried out at the time of storage and later after a year of storage. It was found that after one year of storage, the enzyme retained 95% PCR amplification ability as compared to the one before storage of the same batch as shown in Figure 5b.

Comparison of PCR performance

PCR was performed with 2 U of each *Pca*-Pol, *Pfu* and *Taq* DNA polymerases in their respective PCR buffers. It was found that all these three enzymes produced the same amount of the amplified product and no amplification was observed in the absence of the enzyme (Figure 6). So the

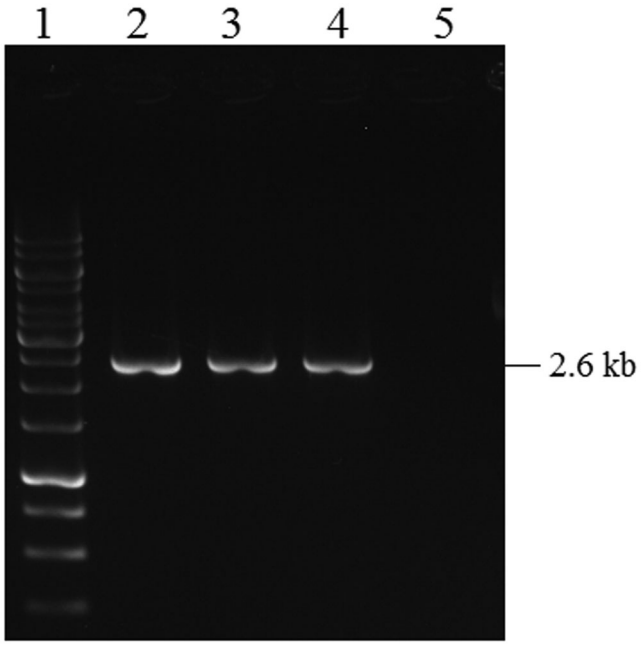

Figure 6. PCR comparison of *Pca*-Pol with other commercial DNA polymerases. Lane 1: DNA ladder; Lane 2: *Taq* DNA polymerase; Lane 3: *Pca*-Pol; Lane 4: *Pfu* DNA polymerase; Lane 5: control (without DNA polymerase).

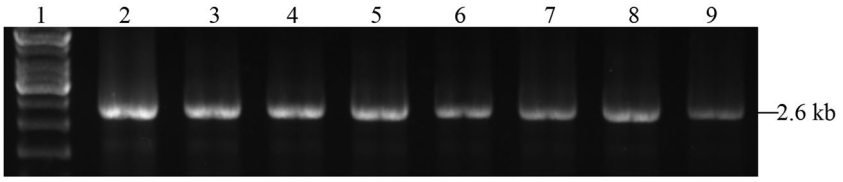

Figure 7. Effect of prolong freeze-thaw on PCR amplification of *Pca*-Pol. Lane 1: DNA ladder; Lane 2: un-thawed; Lane 3: 1 freeze-thaw cycle; Lane 4: 2 freeze-thaw cycles; Lane 5: 3 freeze-thaw cycles; Lane 6: 4 freeze-thaw cycles; Lane 7: 5 freeze-thaw cycles; Lane 8: 6 freeze-thaw cycles; Lane 9: 7 freeze-thaw cycles.

performance of *Pca*-Pol was found comparable to those of *Pfu* and *Taq* DNA polymerases.

Effect of repeated freeze-thaw

The effect of freezing and thawing of *Pca*-Pol on PCR amplification was also investigated. *Pca*-Pol stored under optimal conditions was incubated at room temperature for extended duration (45 min) and then stored at −20 °C overnight. This was repeated and PCR amplification ability of *Pca*-Pol was evaluated. These extended cycles of freeze-thaw affected enzyme activity and the enzyme retained approximately 55% of activity after seven such freeze-thaw treatments as shown in Figure 7.

Discussion

DNA polymerase is an important enzyme used for various applications and processes including routine PCR, real-time PCR, error-prone PCR, site-directed mutagenesis, DNA labeling and sequencing.[12,16] In addition, DNA polymerases are used for applications in synthetic biology and DNA-based computing.[17,18] Here we report gene expression optimization and high-recovery affinity purification of *Pca*-Pol. This enzyme has been shown to amplify DNA fragments up to 7.5 kb and used for DNA labeling application as well.[14]

Heterologous gene expression in *E. coli* is a common method to produce recombinant proteins as it is a well-studied expression host with short doubling time and ability to express recombinant proteins at high rate.[19] In this study, we used *E. coli* BL21 CodonPlus (DE3) RIL strain for expression of *Pca-pol* gene. This strain contains rare-codon transfer RNAs. BL21 strain has also been reported to have better performance in high cell density cultures.[20] *Pca-pol* gene was cloned in plasmid pET28a. pET expression system is based on T7 promoter and requires T7 RNA polymerase from DE3 strain.[21] The resulting recombinant *Pca*-Pol contained N-terminal His-tag. Affinity tags such as His-tag facilitate the process of protein purification by affinity chromatography.[22] This can lead to greater recovery of the required recombinant protein, as in our case, IMAC purification recovered 92% of the total activity in the purified fraction.

Growth parameters are important in governing heterologous gene expression[23] and hence are significant with respect to the yield and cost of production. We optimized the growth phase for IPTG induction, post-induction duration, IPTG concentration and growth temperature. Culture growth phase and growth temperature are among important factors governing protein yield in heterologous gene expression.[24] Optimal expression of *Pca*-Pol was achieved when induced at OD_{600} of 0.6. Post-induction duration was also optimized. Maximum production of *Pca*-Pol was observed after 3 hours of inducer addition. Although biomass kept on increasing with time, but the yield of *Pca*-Pol decreased to almost half after 6 hours of inducer addition. This may be because of proteolysis of the recombinant protein by cellular machinery during growth.[25] Also the amount of other cellular proteins may become higher over the course of time thus decreasing the percentage of the required protein out of total cellular proteins.

Inducer (IPTG) concentration was also optimized for gene expression of *Pca*-Pol. Low concentration of IPTG may lead to inappropriate induction while higher concentrations may cause toxic effects like reduction in cell growth rate and recombinant protein synthesis.[26] Higher concentrations of inducer may also result in inappropriate folding of protein or accumulation of recombinant protein as inclusion bodies.[27] Therefore, it is important to optimize IPTG concentration for optimal expression. We observed maximum expression of *Pca*-Pol at an IPTG concentration of 0.1 mM. No significant increase in gene expression was observed when IPTG concentration was increased further (up to 1 mM).

Growth temperature is of the important factors for optimal bacterial growth and gene expression.[28] To evaluate the effect of temperature on the expression of *Pca*-Pol, cultures were incubated at three different temperatures (20 °C, 30 °C and 37 °C). SDS-PAGE analysis showed that expression occurred at all temperatures. However, densitometric analysis revealed that maximum expression of *Pca*-Pol occurred at 37 °C. *E. coli* can grow over a range of temperature (15 − 42 °C) with an optimal growth at 37 °C. Increase in temperature, up to optimal, results in increasing growth rate of *E. coli*. Also, increase in temperature favors the translational efficiency of *E. coli*[29] as also indicated by our result.

Optimization of expression conditions can have several benefits e.g., equipment can be free for further use along with reduction in the cost of production by reducing the electricity cost as well as long term life of the equipment. The contaminant level and the cost associated with downstream processing is also reduced. In case of *Pca*-Pol, expression optimization and affinity purification can save 87% of the cost when compared to untagged enzyme. Addition of His-Tag is a useful mechanism for affinity purification of recombinant proteins.[22] After optimizing gene expression, we purified His-tagged *Pca*-Pol by IMAC. 92%

of total enzyme activity was recovered (9000 U per liter) in the single-step purified fraction with a 7.4 fold purification. Whereas for untagged *Pca*-Pol, the recovery of the purified enzyme was 60% (nearly 6150 U per liter of the culture).[30] Hence expression optimization and affinity purification improved the yield and recovery of *Pca*-Pol as compared its untagged version. In this current study, we recovered 9000 U of purified *Pca*-Pol per liter of the culture. 2 U of *Pca*-Pol were used for a PCR mixture of 20 µL which produced comparable results as those of *Pfu* and *Taq* DNA polymerases. So, even if a larger volume of PCR reaction mixture is to be used, this preparation of *Pca*-Pol (from one liter of the culture) will be sufficient for more than 1500 PCR reactions.

3′-5′ exonuclease activity of DNA polymerase is important for correcting misincorporated nucleotides and confers proofreading activity to replicate DNA accurately.[31] As reported earlier, untagged *Pca*-Pol possess exonuclease activity.[14] To evaluate the 3′-5′ exonuclease activity of His-tagged *Pca*-Pol, 5′-biotin-labeled primer annealed to a template was used. As expected, His-tagged *Pca*-Pol degraded the annealed primer demonstrating 3′-5′ exonuclease activity. Hence, during DNA synthesis, *Pca*-Pol is expected to have better fidelity as compared to *Taq* DNA polymerase - which does not contain 3′-5′ exonuclease activity.

Although many archaeal DNA polymerases have been cloned and characterized, only a few have made it to commercialization. Apart from PCR efficiency of DNA polymerases, thermal and storage stability are key factors but are generally not reported with other characterization parameters. We analyzed stability of *Pca*-Pol during storage and optimized storage conditions. Glycerol, as a co-solvent in aqueous system, is known to enhance protein stability and prevent aggregation.[32] Likewise, BSA is reported to improve enzyme function by contributing to protein stability.[33] After purification, *Pca*-Pol was stored in buffers with various concentrations of glycerol, BSA and Tween 20 and its PCR amplification ability was evaluated. Highest amplification ability was observed when *Pca*-Pol was stored in 25 mM Tris-Cl pH 8.5 containing 0.1% Tween 20, 0.2 mg/mL BSA and 20% glycerol. Another important factor regarding protein stability during storage is repeated freeze-thaw. Enzymes can lose their activity during freeze-thaw stress.[34] The effect of repeated freeze-thaw cycles on PCR amplification of *Pca*-Pol was evaluated. After seven cycles of repeated freezing (-20 °C, overnight) and thawing (28 °C, 45 min), *Pca*-Pol retained 55% of PCR amplification ability. These prolong freeze-thaw cycles were done to challenge the enzyme to the extreme. When DNA polymerases are used for PCR, under most laboratory conditions, the thawing duration is not so long as tested in our experiment. Even in this extreme case of repeated freeze-thaw, *Pca*-Pol retained 55% residual activity. Also the DNA polymerase solution can be stored in ice, after removing it from −20 °C, to minimize temperature change. So, it is expected that by controlling these factors (i.e., thawing duration and temperature), the activity of *Pca*-Pol will not deteriorate much during storage.

Conclusion

In conclusion, we optimized gene expression and used affinity purification for high recovery (92%) and cost-effective production of *Pca*-Pol. Stability of the enzyme during storage was analyzed. *Pca*-Pol can tolerate freeze-thaw stress well and when stored at −20 °C in suitable buffer, the enzyme was stable over a year.

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ORCID

Syed Farhat Ali http://orcid.org/0000-0001-7854-8488
Naeem Rashid http://orcid.org/0000-0002-3071-5977

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