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CODON OPTIMIZATION, EXPRESSION AND PURIFICATION OF RECOMBINANT *PWO* DNA POLYMERASE IN *E. COLI*

Van Sang Nguyen✉, Thi Thu Huyen Nguyen

Faculty of Biology, VNU University of Science, Vietnam National University, 334 Nguyen Trai Road, Thanh Xuan District, Hanoi, Vietnam

✉To whom correspondence should be addressed. E-mail: nvsangvnu@yahoo.com

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SUMMARY

Pwo DNA polymerase is an enzyme that is widely used in PCR due to its thermal stability and proofreading activity (3′-5′ exonuclease activity) with high accuracy. Optimizing the expression and purification of the protein is crucial to reducing the cost of production for this important enzyme. In this study, the higher expression level of the gene encoding *Pwo* DNA polymerase in bacteria strain *E. coli* BL21(DE3) was achieved by optimizing codon usage. The parameters of the gene encoding *Pwo* DNA polymerase obtained from the optimization: Number of Codons (ENc) = 28; Codon Adaptation Index (CAI) = 0.94; %GC = 48.4%. The codon-optimized gene was cloned into a pET-M expression vector and successfully expressed the *Pwo* DNA polymerase protein with a 6xHis-tag in *E. coli* BL21(DE3) cells. The recombinant protein was purified through a simple and rapid process involving two steps: cell lysis at high temperature combined with affinity chromatography using a Nickel column. The amount of *Pwo* DNA polymerase enzyme obtained from 1 liter of cell culture reached 32 mg. The protein yield in this study is higher than that of previous research while maintaining activity comparable to commercial enzymes. The activity of the *Pwo* DNA polymerase enzyme was tested at concentrations ranging from 60-150 µg/ml which showed to be equivalent or better than the commercially available *Taq* DNA polymerase from Promega. This result actively contributes to the production of the *Pwo* DNA polymerase enzyme and the study of its variants in the future.

Keywords: Enzyme activity assay, PCR, *Pwo* DNA polymerase, recombinant enzyme

INTRODUCTION

Pwo DNA polymerase is a thermostable enzyme derived from the archaeon *Pyrococcus woesei*, demonstrating proofreading activity characteristic of the DNA-polB family (Zhang *et al.*, 2015). The error frequency during DNA replication of *Pwo* DNA polymerase ($1 – 2x10^{-6}$) is approximately 10–20 times lower than that of *Taq* DNA polymerase ($1 – 20x10^{-5}$) (McInerney *et al.*, 2014). Thus, *Pwo* DNA polymerase is well-suited for a variety of applications in PCR amplification and DNA sequencing where high fidelity is crucial (Pavlov *et al.*, 2004). Then, the *Pwo* DNA

polymerase enzyme has been studied for the development of commercial products. Among the various expression host systems, *Escherichia coli* is commonly used as a host for producing foreign genes at high levels in recombinant protein production (Rosano, Ceccarelli, 2014). However, previous studies have indicated particular issues, including poor expression of *Pwo* DNA polymerase and toxicity to *E. coli* BL21(DE3) cells (Dąbrowski, Kur, 1998; Ghasemi *et al.*, 2011). The issues could be attributed to the influence of codons on the expression of *Pwo* DNA polymerase in *E. coli*.

Codon optimization is a strategy to enhance translation efficiency and expression levels of recombinant proteins inside the host cells. Because of an organism's specific codon bias, it often prefers a particular codon for encoding an amino acid. Methods for codon optimization involve converting the DNA sequence using a mathematical programming approach (Fu *et al.*, 2020). Among them, the Codon Adaptation Index (CAI) is widely used to predict the expression level of various genes (Sharp, Li, 1987). The CAI value ranges from 0 to 1, indicating synonymous codons frequency of the gene in the reference set. The CAI index is considered suitable for gene expression in *E. coli*, ranging from 0.8 to 1, with %GC of genes ranging from 30-70% of the total nucleotide content. Therefore, codon optimization is a deciding step in achieving high expression of the Pwo DNA polymerase protein/enzyme in *E. coli*.

Globally, commercially produced DNA polymerase enzymes frequently entail substantial expenses. Hence, codon optimizing the expression and purification of the *Pwo* DNA polymerase enzyme is crucial for cost reduction in production. Our research approach involves in codon optimization, gene cloning, and protein expression of *Pwo* DNA polymerase in the *E. coli* BL21(DE3) strain. This research explored the application of some molecular techniques, such as *de novo* synthetic gene synthesis (Yang *et al.*, 2011), cloning using the PCR method (Hoseini, Sauer, 2015), purification of recombinant protein using the affinity chromatography method (Spriestersbach *et al.*, 2015). The research aims to develop a simple expression, purification, and evaluation process for the efficient production of the *Pwo* DNA polymerase enzyme. Further studies involve modifying *Pwo* DNA polymerase for commercialization and its application across various biological targets.

MATERIALS AND METHODS

Materials

The *E. coli* DH5α strain was utilized for gene cloning, while the *E. coli* BL21(DE3) strain was employed for expressing the recombinant protein. Subcloning and expression of *Pwo* DNA polymerase were carried out using the plasmid pET-M expression vector, a variant of pET-32a with the removal of the *S-tag* and thioredoxin (*TrxA*) gene sequence. Gene and primers for cloning were chemically synthesized by Phusa Genomes (Can Tho, Vietnam). The LB broth and reagents for preparing buffer solutions were procured from reputable suppliers such as Merck, Thermo Scientific™, and Sigma.

Codon optimization

The gene sequence in this study was optimized for expression of the gene in *E. coli* based on the amino acid sequence of

the DNA-dependent DNA polymerase protein from *Pyrococcus woesei* (GenBank accession number AAB67984) using the GenSmart Codon Optimization tool (GenScript Biotech Corporation). The gene of 2328 bp was artificially synthesized by Genscript and cloned into pUC19 to generate plasmid pUC19-Pwo.

PCR and cloning of *Pwo* gene into vector pET-M

The reaction amplifying the *Pwo* DNA polymerase gene was conducted with a volume of 50 µl, including PCR components of 10 µl HF buffer (Thermo Scientific™), 1.5 µl forward primer 10 µM (Pwo-BamHI 5'- GC GGA TCC ATT CTG GAT GTG GAT TAT ATC AC -3'), 1.5 µl reverse primer 10 µM (Pwo-EcoRI 5'- CG GAA TTC TTA GCT TTT CTT AAT GTT CAG C -3'), 0.2 mM dNTPs mix, 2.5 U enzyme Phusion Hotstart II DNA Polymerase (Thermo Scientific™) and 1.5 µl template 10 ng/µl (pUC19-Pwo). The PCR temperature cycle was as follows: 30 seconds of initial denaturation at 98°C; 35 cycles of 15 seconds at 98°C, 15 seconds at 60°C, and 1 minute at 72°C; and 5 minutes of final extension at 72°C.

The gene and the pET-M plasmid were digested with the *Bam*HI and *Eco*RI enzymes in a solution containing Fast Digest Buffer. The digestion reactions were incubated at 37°C for 2 hours. After digestion, the gene and expression vector were purified using the MEGAquick-spin™ Total Fragment DNA Purification Kit (iNtRon, South Korea) and then ligated together by T4 DNA ligase. The mixture was incubated with *E. coli* DH5α competent cells before heat shock transformation and spread over the surface of an LB-ampicillin agar plate (100 µg/ml).

Screening and sequencing of recombinant DNA clones

Transformants carrying the recombinant plasmid were verified using the PCR with the described components and thermal cycles above. The extracted plasmids by GeneJET Plasmid Miniprep Kit (Thermo Scientific™) were used for sequencing of the inserted gene in the company 1st Base (Malaysia) using the Sanger method.

Expression of Pwo DNA polymerase in *E. coli* BL21(DE3)

The recombinant vector pET-M-Pwo was inputted into *E. coli* BL21(DE3) cells. A single bacterial colony was cultured overnight in a 10 ml LB medium supplemented with 100 µg/ml ampicillin. After transferring the entire 10 ml overnight culture to 1 L LB media with 100 µg/ml ampicillin, the culture was agitated at 37°C for approximately 5–6 hours, or until the bacterial density at OD_{600} reached 0.6–0.8. The cells were induced by IPTG at 0.3 mM concentration for 16h. The culture medium was centrifuged at 6000 rpm at 4°C for 10 minutes to collect the cell pellets, which were then stored at -80°C.

Purification of recombinant protein

The cell pellets from 1 L of the cell culture were resuspended in 100 ml buffer A (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 5 mM imidazole) and heated at 90°C for 30 minutes. Insoluble components from the heated solution were removed by centrifugation at a high speed of 13,000 rpm at 4°C for 30 minutes. Then, the supernatant was purified using a nickel resin column (Ni-NTA, Thermo Scientific™). Buffer B (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 30 mM imidazole) was used to wash the

column. Buffer C (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 500 mM imidazole) was used to elute and recover the recombinant protein from the column. The eluted solution was dialyzed in buffer D (20 mM Tris-HCl, pH 8.0, 200 mM NaCl) at 4°C for 3 hours. The buffer was changed three times. The protein solution obtained was measured for concentration using the Bradford method before being stored at -80°C.

Enzyme activity of *Pwo* DNA polymerase

Enzyme activity was determined using the PCR method. The enzyme was diluted in a storage buffer. A plasmid template (2.5 ng/µl) was used as a PCR component for amplifying gene fragments of sizes 235, 482, 734, 1117, and 2036 bp. Each amplified reaction had a volume of 20 µl, consisting of 1 µl diluted enzyme, 2 µl buffer (containing 30 mM $MgCl_2$), 0.5 µl each primer, forward and reverse (10 µM), and 0.4 µl dNTPs (10 mM). The thermal cycling conditions for the reactions included the first denaturation step at 95°C for 2 minutes; 35 cycles of steps: denaturation (15 seconds at 95°C), primer annealing (15 seconds at 57°C), and extension (15–120 seconds at 72°C depended on the target gene sizes); then a last extension step at 72°C for 5 minutes. The results of PCR amplification were evaluated on a 1% agarose gel in 1X TAE buffer.

RESULTS

Analysis of codon usage

Initially, the gene sequence encoding the *Pwo* DNA polymerase protein comprised 57 codons (excluding CCG encoding proline and CGC, CGA, CGG encoding arginine). After the processing, the number of codons was reduced to 28, as indicated in Table 2. Condon usage indices were compared between the original sequence and optimized sequence as follows: Codon Adaptation Index (CAI) = 0.98 (pre-optimization CAI = 0.53); Effective Number of Codons (ENc) = 28 (pre-optimization ENc = 57); %GC content = 48.42 (pre-optimization %GC = 39.3). The gene (2328 bp) was synthesized and inserted into pUC19.

Construction of recombinant expression vector

By PCR the fragment of the *Pwo* gene (2344 bp) was successfully amplified from pUC19-Pwo (Figure 1a). The PCR products and plasmid pET-M were double digested by *Bam*HI and *Eco*RI (Figure 1b) and then ligated together. After transformation, bacterial colonies carrying the pETM-Pwo vector were screened PCRs using T7-promoter and T7-terminator primers.

In Figure 2a, lanes of Pwo2 and Pwo3 colonies exhibited a bright band with a size of 2599 bp, corresponding to the size of the *Pwo* region expanding from T7-promoter to T7-terminator. The lane of the Pwo1 colony showed a band with a size of 266 bp corresponding to the region from T7-promoter to T7-terminator of pET-M. Consequently, the Pwo1 colony lacks the recombinant plasmid, whereas the Pwo2 and Pwo3 colonies carried the Pwo gene. Plasmid from these two bacterial colonies was extracted (Figure 2b) then the foreign genes were verified by sequencing.

The sequence alignment result (Figure 3) revealed that the gene encoding *Pwo* DNA polymerase has been accurately inserted into the open reading frame

containing a 6xHis-tag and the thrombin cleavage sequence of the pET-M plasmid. The sequence showed the absence of any substitutions or frame-shift mutations when compared to the original sequence. Thus, these colonies carrying recombinant plasmid were used for the expression of *Pwo* DNA polymerase.

Table 1. Components of condon usage in optimized gene sequence.

aa	Codon	Number	Fraction	aa	Codon	Number	Fraction
Phe	UUU	26	1,93	**Asn**	AAC	19	2
	UUC	1	0,07	**Lys**	AAA	74	1,8
Leu	CUG	67	6		AAG	8	0,2
Ile	AUU	62	2,62	**Asp**	GAU	37	1,95
	AUC	9	0,38		GAC	1	0,05
Met	AUG	10	1	**Glu**	GAA	87	1,98
Val	GUG	52	4		GAG	1	0,02
Pro	CCG	36	4	**Cys**	UGC	4	2
Thr	ACC	31	4	**Trp**	UGG	11	1
Ala	GCG	44	4	**Arg**	CGU	1	0,13
Tyr	UAU	44	1,91		CGC	44	5,87
	UAC	2	0,09	**Ser**	AGC	25	6
His	CAU	13	1,86	**Gly**	GGC	50	4
	CAC	1	0,14	**TER**	UAA	1	3
Gln	CAG	15	2				

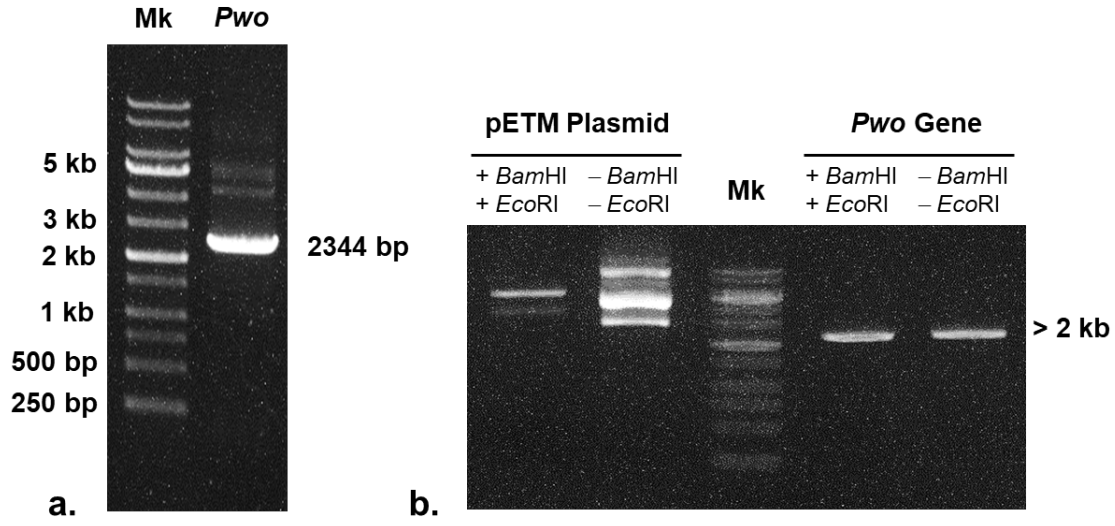

Figure 1. Analysis of PCR products amplified the gene encoding *Pwo* DNA polymerase (a) and pET-M, Pwo digested products (b) on 1% agarose gels. Mk: 1 kb marker (iNtRon).

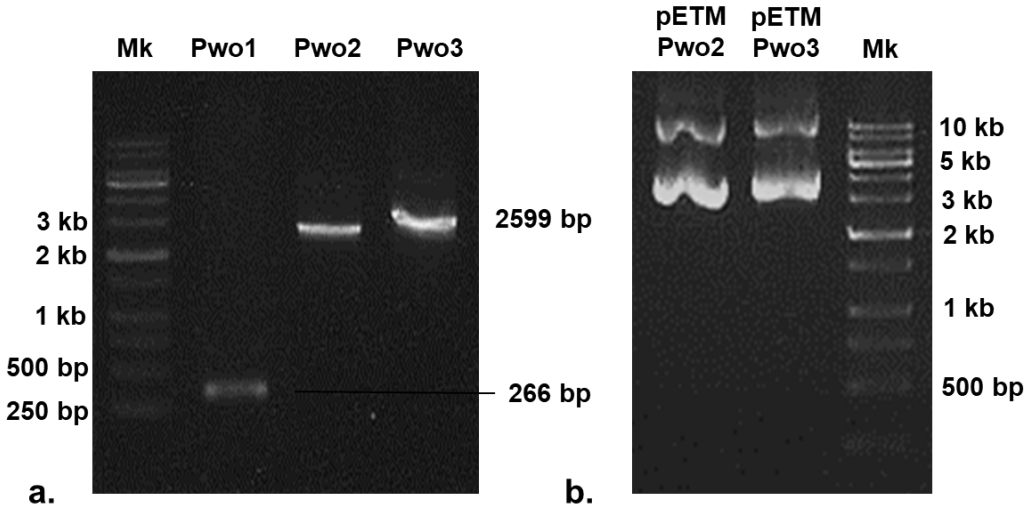

Figure 2. Analysis of PCR colony products (a) and plasmid pETM-Pwo clones 2, 3 (b) by electrophoresis on 1% agarose gel. Mk: 1 kb marker (iNtRon).

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P.woesei            ---------------MILDVDYITEEGKPVIRLFKKENGKFKIEHDRTFRPYIYALLRDD
Pwo-recombinant     MHHHHHHSSGLVPRGSILDVDYITEEGKPVIRLFKKENGKFKIEHDRTFRPYIYALLRDD
                                   ******************************************

P.woesei            SKIEEVKKITGERHGKIVRIVDVEKVEKKFLGKPITVWKLYLEHPQDVPTIREKVREHPA
Pwo-recombinant     SKIEEVKKITGERHGKIVRIVDVEKVEKKFLGKPITVWKLYLEHPQDVPTIREKVREHPA
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P.woesei            VVDIFEYDIPFAKRYLIDKGLIPMEGEEELKILAFDIETLYHEGEEFGKGPIIMISYADE
Pwo-recombinant     VVDIFEYDIPFAKRYLIDKGLIPMEGEEELKILAFDIETLYHEGEEFGKGPIIMISYADE
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P.woesei            NEAKVITWKNIDLPYVEVVSSEREMIKRFLRIIREKDPDIIVTYNGDSFDFPYLAKRAEK
Pwo-recombinant     NEAKVITWKNIDLPYVEVVSSEREMIKRFLRIIREKDPDIIVTYNGDSFDFPYLAKRAEK
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P.woesei            LGIKLTIGRDGSEPKMQRIGDMTAVEVKGRIHFDLYHVITRTINLPTYTLEAVYEAIFGK
Pwo-recombinant     LGIKLTIGRDGSEPKMQRIGDMTAVEVKGRIHFDLYHVITRTINLPTYTLEAVYEAIFGK
                    ************************************************************

P.woesei            PKEKVYADEIAKAWESGENLERVAKYSMEDAKATYELGKEFLPMEIQLSRLVGQPLWDVS
Pwo-recombinant     PKEKVYADEIAKAWESGENLERVAKYSMEDAKATYELGKEFLPMEIQLSRLVGQPLWDVS
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P.woesei            RSSTGNLVEWFLLRKAYERNEVAPNKPSEEEYQRRLRESYTGGFVKEPEKGLWENIVYLD
Pwo-recombinant     RSSTGNLVEWFLLRKAYERNEVAPNKPSEEEYQRRLRESYTGGFVKEPEKGLWENIVYLD
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P.woesei            FRALYPSIIITHNVSPDTLNLEGCKNYDIAPQVGHKFCKDIPGFIPSLLGHLLEERQKIK
Pwo-recombinant     FRALYPSIIITHNVSPDTLNLEGCKNYDIAPQVGHKFCKDIPGFIPSLLGHLLEERQKIK
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P.woesei            TKMKETQDPIEKILLDYRQKAIKLLANSFYGYYGYAKARWYCKECAESVTAWGRKYIELV
Pwo-recombinant     TKMKETQDPIEKILLDYRQKAIKLLANSFYGYYGYAKARWYCKECAESVTAWGRKYIELV
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P.woesei            WKELEEKFGFKVLYIDTDGLYATIPGGESEEIKKKALEFVKYINSKLPGLLELEYEGFYK
Pwo-recombinant     WKELEEKFGFKVLYIDTDGLYATIPGGESEEIKKKALEFVKYINSKLPGLLELEYEGFYK
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P.woesei            RGFFVTKKRYAVIDEEGKVITRGLEIVRRDWSEIAKETQARVLETILKHGDVEEAVRIVK
Pwo-recombinant     RGFFVTKKRYAVIDEEGKVITRGLEIVRRDWSEIAKETQARVLETILKHGDVEEAVRIVK
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P.woesei            EVIQKLANYEIPPEKLAIYEQITRPLHEYKAIGPHVAVAKKLAAKGVKIKPGMVIGYIVL
Pwo-recombinant     EVIQKLANYEIPPEKLAIYEQITRPLHEYKAIGPHVAVAKKLAAKGVKIKPGMVIGYIVL
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P.woesei            RGDGPISNRAILAEEYDPKKHKYDAEYYIENQVLPAVLRILEGFGYRKEDLRYQKTRQVG
Pwo-recombinant     RGDGPISNRAILAEEYDPKKHKYDAEYYIENQVLPAVLRILEGFGYRKEDLRYQKTRQVG
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P.woesei            LTSWLNIKKS
Pwo-recombinant     LTSWLNIKKS
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Figure 3. Amino acid sequence alignment of recombinant form with the wild type form of Pwo DNA polymerase. The amino acid chain contains 6 histidines (highlighted in red), and the cleavage site sequence of thrombin (highlighted in blue). The mark "*" indicates the similarity between the amino acids of the two sequences.

Expression and purification of the recombinant protein

The recombinant vector pETM-Pwo exhibited the ability to autonomously replicate within the genetic system of the *E. coli*. The expression of *Pwo* DNA polymerase in the recombinant vector was controlled by the T7-RNA polymerase promoter present in the pETM vector system. The SDS-PAGE analysis (Figure 4) revealed that a prominent protein band, approximately 90 kDa in size was produced in *E. coli* BL21(DE3) cells cultured in medium with 0.3 mM IPTG, while was absented in the uninduced cells (Figure 4). Thus, the *Pwo* DNA polymerase protein was expressed in *E. coli* BL21(DE3) cells.

Subsequently, the cells were lysed at high temperatures to release recombinant protein for protein purification.

The SDS-PAGE gel electrophoresis of protein purification (Figure 4) demonstrated that the high temperature effectively disrupted the cells for recombinant protein extraction. The recombinant protein with 6xHis-tag was further purified through a Ni-NTA resin column. The proteins without the 6xHis-tag, which could not bind to Ni^{2+} ions, were washed away during the purification process. Finally, the *Pwo* DNA polymerase protein yielded 32 mg from a liter cell culture.

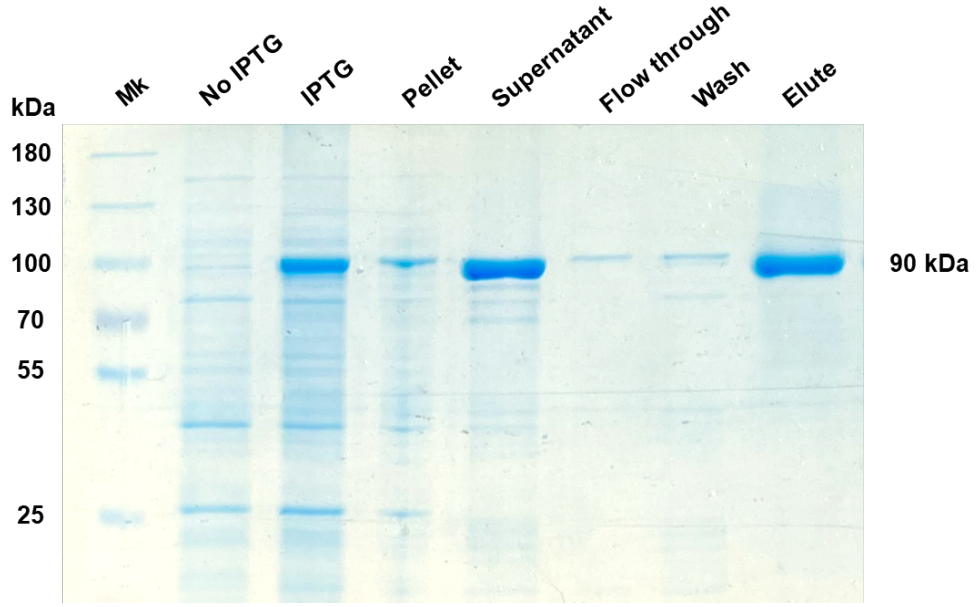
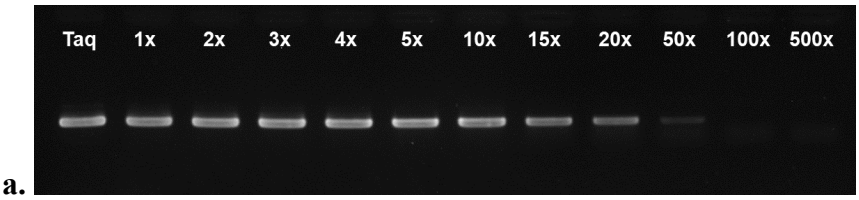


Figure 4. SDS-PAGE analysis of *Pwo* DNA polymerase protein expression in *E. coli* BL21(DE3) and the enzyme purification. Mk: protein marker (Thermo Scientific).

Optimization of enzyme concentration in PCR

The activity of the *Pwo* DNA polymerase enzyme was evaluated by PCRs amplifying DNA fragments of 482 bp. A dilution series of enzymes, ranging from the original concentration of 0.3 mg/ml, was used for PCR tests. The results of PCR amplification were analyzed on a 1% agarose gel (Figure 5a). As the dilution ratio increased, the enzyme's activity gradually decreased, demonstrating that the enzyme's activity is dependent on its concentration. The activity of *Pwo* DNA polymerase has remained even at a concentration as low as 6 µg/ml (lane 50x).

The relationship between band intensity and enzyme was performed in Figure 5b. Lanes 2x, 3x, 4x, and 5x of the *Pwo* DNA polymerase enzyme exhibited bright bands with an intensity higher than that of the *Taq* DNA polymerase enzyme from Promega (Taq). Specifically, the mean gray values of the predicted bands in these lanes were 19.8, 20.2, 19.5, and 19.4, respectively. Thus, the optimal activity of the *Pwo* DNA polymerase enzyme is observed at concentrations ranging from 60 to 150 µg/ml.



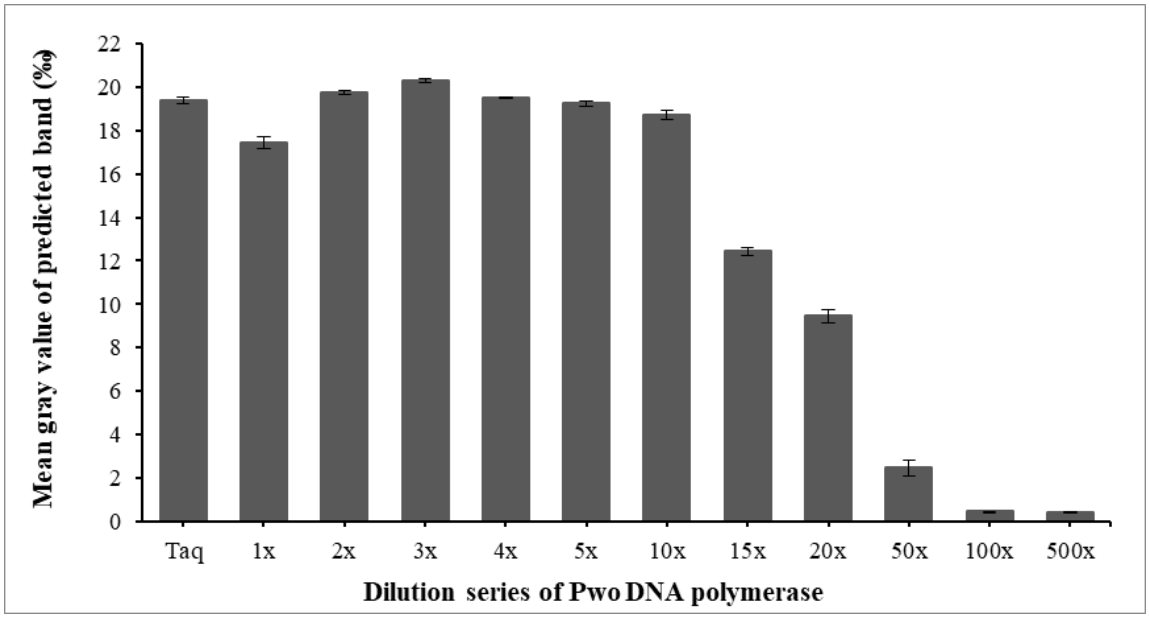


Figure 5. Effect of enzyme concentration on the activity of *Pwo* DNA polymerase enzyme in PCR. (a) Electrophoresis of PCR products in a 1% agarose gel, (b) the relationship between band intensity and Pwo DNA polymerase concentration, and the mean values (± SD) of predicted bands were measured by ImageJ (Rasband, 2011). Taq: enzyme *Taq* DNA polymerase (Promega); the initial enzyme concentration is 300 µg/ml (1x), and it is utilized to prepare dilutions ranging from 2x to 500x concentrations, including 150 µg/ml (2x), 100 µg/ml (3x), 75 µg/ml (4x), 60 µg/ml (5x), 30 µg/ml (10x), 20 µg/ml (15x), 15 µg/ml (20x), 6 µg/ml (50x), 3 µg/ml (100x), and 0.6 µg/ml (500x).

Dependence of *Pwo* DNA polymerase enzyme on extension time in PCR

The optimal concentration of enzyme *Pwo* DNA polymerase, equivalent to 0.1 mg/ml was used for PCR reactions amplifying gene fragments of various sizes: 235, 482, 734, 1117, and 2036 bp, respectively. The extension time for gene fragments was compared between *Pwo* DNA polymerase and *Taq* DNA polymerase from Promega, as shown in Figure 6. For sizes under 1 kb, the *Pwo* DNA polymerase enzyme exhibits target gene amplification rates equivalent to the commercial enzyme. For sizes larger than 1 kb, the average amplification rate of the enzyme is 1 minute per 1 kb.

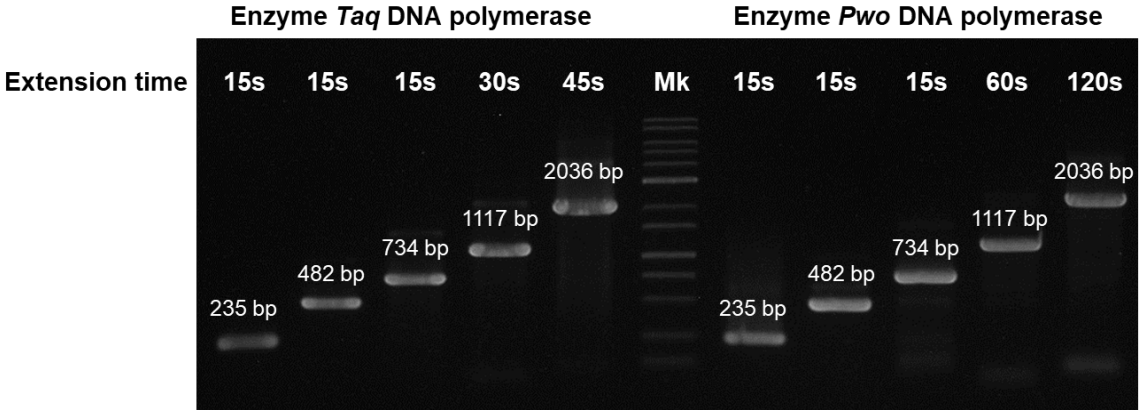


Figure 6. The impact of the extension times on the *Pwo* DNA polymerase enzyme in comparison with the *Taq* DNA polymerase enzyme in PCRs.

DISCUSSION

We optimized the codon usage of the *Pwo* DNA polymerase gene to ensure recombinant protein expression compatibility with *E. coli* host cells. The optimization process eliminated at least 7 rare codons in *E. coli*, namely AGG, AGA, CGA, CGG encoding arginine; AUA encoding isoleucine; CUA encoding leucine, and CCC encoding proline. Rare codons play a significant role in forming secondary RNA structures for stability and interaction with ribosomes, supporting protein folding during translation termination (Kane, 1995). Thus, they have the potential to reduce both the quantity and quality of synthesized proteins, which should be excluded. In our research, the achieved sequence was evaluated using two critical indices: a CAI (codon-adapted index) reached 98%, exceeding the threshold of 90% (Sharp, Li, 1987); and an ENc (effective number of codons) was 28, in proximity to the expected value of 25 codons for an *E. coli* gene (Wright, 1990).

In research on thermostable DNA polymerases, the purification process using high temperatures is particularly popular and effective. The temperature employed ranges from 75°C to 100°C, and incubation times can extend up to 1 hour (Ghasemi *et al.*, 2011; Heo, Kim, 2013; Konovalova *et al.*, 2017). However, prolonged incubation at high temperatures may affect the stability of the enzyme. Therefore, in this study, we optimized the temperature to 90°C for 30 minutes. Following the purification process, 32 mg of active *Pwo* DNA polymerase protein was obtained from 1 L of the culture medium. This outcome demonstrates a notable increase compared to the 26 mg/1L reported by (Dąbrowski, Kur, 1998) and is twice the amount obtained by (Ghasemi *et al.*, 2011), which was 14 mg/1L. These results indicated effective production of *Pwo* DNA polymerase can be achieved using codon optimization and a simple purification process.

CONCLUSION

The codon usage of the gene encoding *Pwo* DNA polymerase has been optimized for recombinant protein expression in *E. coli*, resulting in a Codon Adaptation Index (CAI) of 0.98. The cloned gene was inserted

into the pET-M vector and expressed in large quantities as a recombinant protein in *E. coli* BL21(DE3) bacteria. Combination of heat treatment and nickel affinity chromatography column, *Pwo* DNA polymerase was successfully purified the yield reached 32 mg/1L of the culture medium. The recombinant enzyme exhibited good activity in elongating DNA up to the investigated size of 2036 bp.

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