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Cloning and Expression of *Bst* DNA Polymerase I Gene in *E. coli* BL21

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Abstract

Introduction: DNA Polymerases, in addition to being indispensable in replication and repair, are also very useful in a number of molecular biology techniques such as DNA amplification, site-directed mutagenesis, DNA sequencing, different kinds of PCR, loop-mediated isothermal amplification (LAMP), etc. After the invention of PCR, efforts have been made to focus on the identification and isolation of thermo-tolerant enzymes that amplify DNA efficiently at high temperatures.

Materials and methods: In this study, *Geobacillus stearothermophilus* strain 10 was selected for the cloning of *Bst* DNA Pol I - encoding gene. Following DNA extraction from the bacterium, PCR was carried out to amplify the *pol A* gene using designed primers and to clone via pET32a expression vector followed by transfer to the heterologous *E.coli* BL21 host. The cloned gene was expressed by induction with IPTG and the resultant protein purified by IMAC column.

Results: The activity of the functional fragment was assessed by LAMP and showing a relatively high DNA amplification ability in comparison with commercial *Bst* DNA Polymerase which is usually used in this amplification protocol.

Discussion and conclusion: This study found that Klenow fragment of recombinant *Bst* DNA Pol I can amplify *uidA* gene in *E. coli* ATCC25923 during the LAMP reaction. Separation of two fragments of the enzyme can improve the activity of Klenow fragment of enzyme in LAMP.

Key words: *Bst* DNA Polymerase I, Cloning, Expression, LAMP, PCR

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Introduction

Definitions of thermophilic organisms vary in the literature, but they have been described as those which are able to grow at temperatures above 60°C.

Many biotechnological processes require high temperatures in order to function and/or to produce adequate yields. To name one application, Polymerase Chain Reaction (PCR) is the best known and a revolutionary example of the utilization of thermophilic 'technology' (1).

Taq polymerase was identified from *Thermus aquaticus* isolated from Yellowstone National Park in Montana, USA. Presently, DNA polymerase I from *T. aquaticus* (*Taq* Polymerase) is one of the well-known enzymes among thermostable DNA polymerases. In 1985, PCR (Polymerase Chain Reaction) technology using the Klenow fragment of DNA Polymerase I from *Escherichia coli* was used but the process was tedious and expensive with such mesophilic DNA Polymerases, so efforts were directed at finding a thermostable enzyme which was able to amplify DNA in high temperatures. DNA polymerase from *Thermus thermophilus* (Tth polymerase) was also developed as a commercial product in the early age of the PCR. A specific property of Tth polymerase is that it has a distinct Reverse Transcriptase (RT) activity, and a single-tube RT-PCR method was eventually developed with this enzyme (2).

DNA Polymerases from *Geobacillus stearothermophilus* strain *10* is Bst DNA Polymerase which is widely used in isothermal reaction such as LAMP but the detailed information of commercial Bst DNA polymerase is unpublished and well protected by patents, which results in the high price of commercial kits (5).

Bst DNA polymerases possess a strong strand displacement activity; however, they are not stable at temperatures above 70°C. Enzymes used in PCR (e.g., *Taq* DNA polymerase) possess high thermostability and robust polymerase activity but do not exhibit a strong strand displacement activity and are therefore not suitable for isothermal amplification methods such as LAMP (6).

LAMP is an established nucleic acid amplification method offering rapid, accurate, and cost-effective diagnosis of infectious diseases. This technology has been developed into commercially available detection kits for a variety of pathogens including bacteria and viruses. The current focus on LAMP methodology is to develop a diagnostic system for fatal tropical diseases to be employed in resource-limited laboratories in developing countries. The combination of LAMP and novel microfluidic technologies such as Lab-on-a-chip may facilitate the realization of genetic point-of-care testing systems to be used by both developed and developing countries in the near future (7).

In this study, cloning of *Bst* DNA Polymerase I gene from *Geobacillus stearothermophilus* strain 10 was carried out in *E.coli* BL21 and the resultant recombinant protein was assessed for activity in the LAMP protocol.

Materials and Methods

Bacterial strains and growth conditions: *Geobacillus stearothermophilus* strain 10 was grown on nutrient agar at 58˚C for 24 h. *E. coli* BL21 (DE3) was cultured in nutrient broth medium overnight at 37˚C. Following transformation, *E .coli* colonies carrying the recombinant vector were selected on LB medium with 50 µg/µl ampicillin**.**

Cloning of *G. stearothermophilus* strain 10 *polA* gene in pET32a (+): Genomic DNA from *G. stearothermophilus* strain 10 was extracted by the SET buffer method (8). The primers *Bam*HI 5´ (ATAT AGATCT

GGAAGCATCCGAGGAGGG) 3' and *Bgl*II 5´ (ATAT GGATCC TTTCGCATCATACCACGTCG) 3' were designed based on the *G. stearothermophilus polA* gene sequence deposited in the GenBank. Primers included a *Bam*HI restriction site at the 5´ end and a *Bgl*II restriction site at the 3´ end of the gene (underlined). The *polA* gene was amplified from *G. stearothermophilus* strain 10 by PCR in a reaction containing 0.01 µg/µl template DNA, 0.5 µM of each primer, 5 µl of 10X Taq polymerase buffer, 2 mM of MgCl2, 0.2 mM of each dNTP, 0.5 µl (1 unit) of Taq polymerase in 50 µl volume. Amplification was performed in a thermal cycler (PeqLab, UK) and initiated with a primary denaturation step at 96˚C for 5 min, followed by 38 cycles of 94˚C for 1min, 64.7˚C for 50 sec and 72˚C for 90 sec and 10 min for final extension. PCR product was separated on 2% agarose gel and visualized by SyBr Gold dye (Invitrogen, USA). Following the initial confirmation, the amplicon was purified with DNA extraction kit (Fermentas, Lithuania), then digested with *BamHI* (Sinaclon, Iran) and *BglII* (Fermentas, Lithuania). The same digestion reaction was carried out on the expression vector pET32a (+) (Novagen). Ligation (overnight, at room temperature) was done with T4 Ligase (Fermentas, Lithuania) after which the resulting plasmid containing *polA* gene sequences, was used to transform *E. coli* (BL21). All reactions such as digestion, ligation and transformation procedures were performed according to the manufacturer's instructions.

To confirm the presence of the recombinant plasmid in transformed cells, the plasmid was extracted from the cells by alkaline lysis method (9) and analyzed by PCR. The amplicon was sequenced (Microgen, S. Korea) and the sequence compared with its GenBank origin for confirmation. A pair of primers (forward primer 5'(CCGGGAGAAAGTGGTCGCTT)3' and reverse primer 3' (TCGTATGCACCTTCTTTGTATCGG) 5' were also designed for the central region of *polA* gene to obtain complete gene sequencing following which the results were compiled and used for BLAST analysis.

Expression of the recombinant *polA* gene: The recombinant plasmids containing the *polA* gene insert, were used to transform *E.coli* Bl21(DE3). To assess the expression of *polA*, positive colonies were cultured in LB medium containing 50 µg/µl ampicillin following which 1 mM IPTG (Isopropyl-beta-D-thiogalacto-pyranoside) was added as an inducer to the medium with OD = 0.59 and samples were collected before induction and 2, 4, 6 and 7 hours after induction. The cells were harvested, treated with lysis buffer (sodium chloride 300 mM, sodium phosphate 50 mM and imidazole 10 mM) and centrifuged after which the supernatant was used for SDS-PAGE analysis. Gels were stained with Coomassie Blue R250.

Purification and verification of the recombinant protein activity: Cells were lysed and the recombinant protein purified by Ni-NTA column as specified by the manufacturer's instructions (Biorad, USA). The purified protein was subsequently analyzed by SDS-PAGE (Fig 2).

To verify the polymerase activity of the recombinant protein, LAMP was carried out (10). In this reaction, two pairs of primers (unpublished data) were used to amplify *uidA* gene in *E. coli* ATCC25923. To confirm the success of LAMP using the recombinant *Bst* DNA Pol I protein, two tests were carried out: 1) electrophoresis of the resulting amplicon on 1.5% agarose gel and 2) addition of 1µl SyBr Gold to the amplicon and observation of samples under the UV light for fluorescence.

Results

Cloning of the *polA* gene: Following DNA extraction from *G. stearothermophilus* strain 10, amplification of the Bst DNA Pol gene sequences was carried out with PCR using primers with restriction endonuclease recognition sites. The amplicon was represented by a 2637 bp band on a 1.5% agarose gel which was the expected size of the desired fragment (Fig.1). The correct direction of the gene in the expression vector was also ensured using two different endonuclease sites. Recombinant plasmids were extracted from positive E.coli BL21 clones, PCR with cloning primers were performed and the results confirmed the presence of the *Bst* Pol I sequences.

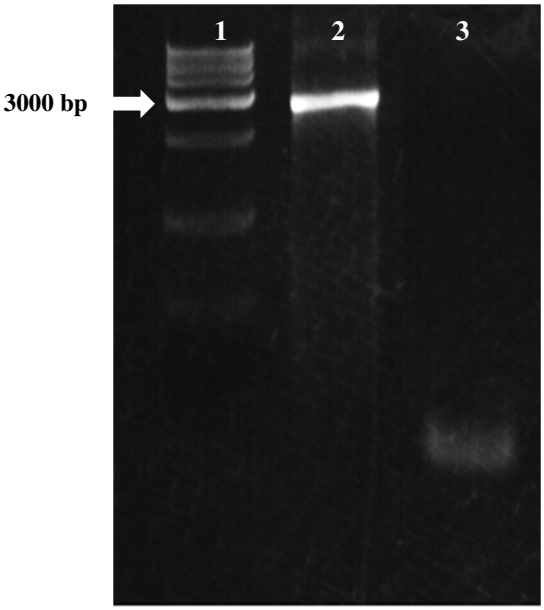

Fig. 1- Results from gene amplification with cloning primers. 1) Molecular weight marker 2) PCR product 3) negative control

Expression and purification of the recombinant protein: The recombinant protein expression from the cloned gene sequences was induced by the addition of 1 mM IPTG. The optimum growth period after addition of IPTG was determined to be 6 hrs which results from SDS-PAGE. This protein is a 99.69KD polypeptide; however, after purification by Ni-NTA affinity column, two band of 64 KDa and 35.69 KDa were detected which were most likely due to enzymatic digestion (Fig 2).

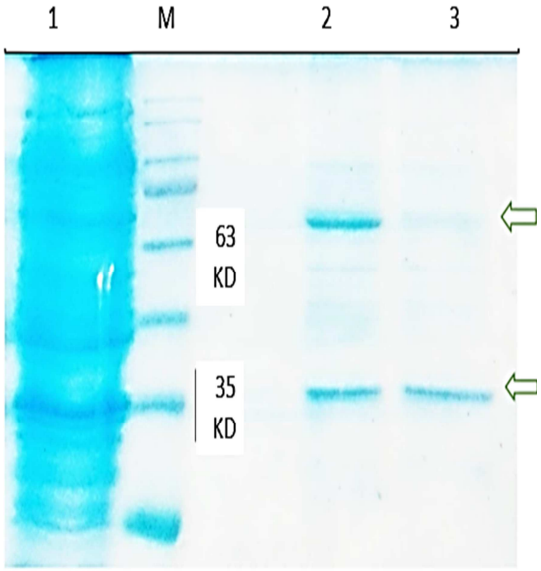

Fig. 2- SDS-PAGE analysis of recombinant *Bst* DNA Pol 1- supernatant fraction after induction for 7h, M-molecular weight marker 2, 3- Sample after elution from IMAC column.

Verification of *Bst* Pol DNA enzymatic activity: One of the fractions eluted from the column containing both 64 KDa and 35.69 KDa was then used in the LAMP reaction to evaluate enzyme activity following which agarose gel electrophoresis showed ladder-like pattern of LAMP amplification. In parallel, the addition of 1µl SyBr Gold dye to the remainder of the amplified sample resulted in a visual increase in fluorescence under the UV irradiation, indicative of DNA amplification. The fluorescence in the negative control, in which DNA was in a reaction excluding the enzyme fraction, was negligible. Furthermore, with fractions that have only 35.69 KDa fraction of the enzymatic activity, the result from LAMP was negative (Fig 5).

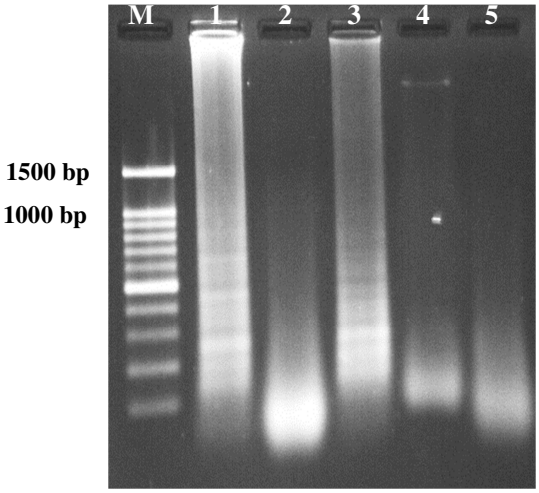

Fig. 3- M) Molecular weight marker 100 bp, 1) positive control with *Bst* DNA Polymerase (NEB) 2) reaction with 1µl recombinant Bst DNA polymerase 3) reaction with 2µl enzyme 4) reaction with 10µl 5) negative control

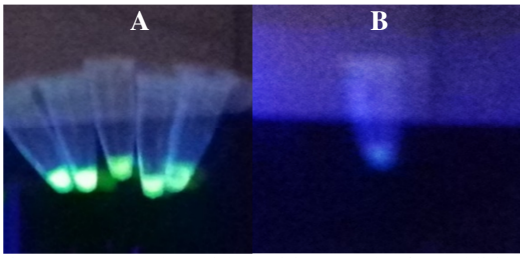

Fig. 4- A) Samples with positive result after addition of 1 µl SyBr Gold, B) Sample with negative result after addition of 1 µl SyBr Gold

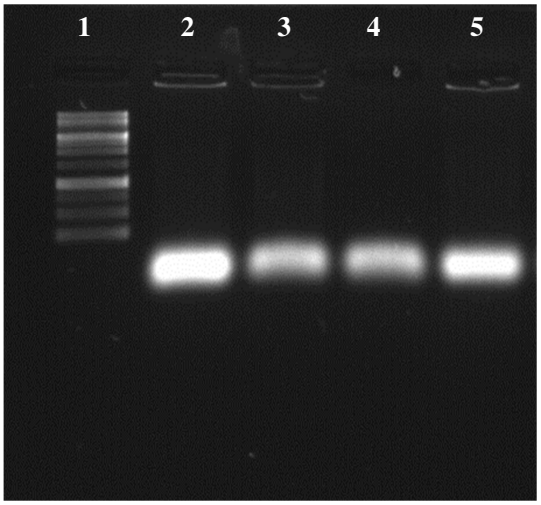

Fig. 5- 1) Molecular weight marker 250 bp. 2, 3 ,4 ,5) lamp with 35.69 KDa fragment of *Bst* DNA Polymerase I

Discussion and Conclusion

Recent developments relating to DNA polymerases have facilitated new genetic analysis approaches that are different from PCR or PCR-related techniques. Simultaneous and non-cyclic temperature involvement in amplification of nucleic acids is more convenient and practical than PCR and hence development of this type of method is being pursued (11).

In PCR, expensive material and instruments as well as longer reaction periods and the presence of a wide range of inhibitors impede the detection of microorganisms while LAMP is considered to be a suitable alternative (12). This amplification is a one-step reaction that amplifies a target DNA sequence with high sensitivity and specificity under isothermal conditions. In the initial phase of development, the application of LAMP has been extended to the detection of many kinds of pathogens causing food-borne diseases. LAMP kits for detecting *Salmonella*, *Legionella*, *Listeria*, verotoxin-producing *Escherichia coli*, and *Campylobacter* have been commercialized. This protocol exhibits less sensitivity to inhibitory substances present in biological samples compared to PCR. This robustness of LAMP against inhibitors can contribute to the time saved and the low-cost required for sample processing steps (13).

In Iran, efforts have been directed at isolating, cloning, expressing, sequencing, determining of the 3D structure of *polA* gene from native thermophilic bacteria by Sadeghizadeh and his colleagues (3, 4). However, isothermal activity of the recombinant enzyme in LAMP was not reported.

Bst DNA Pol I enzyme is being used for the isothermal amplification of sequences which make this detection possible. In this study, the *polA* gene from *G. stearothermophilus* strain 10 has been successfully isolated, cloned and expressed

in a heterologous host (*E.coli* BL21) resulting in the production of the active Klenow fragment of the enzyme (the 64 KDa fragment) in the purified protein fraction as well as a 33 KDa peptide. In the past, there have also been reports on the enzymatic digestion of the Bst DNA polymerase polypeptide (14). The larger fragment of this recombinant Bst Polymerase is now commercialized under the name IsoTherm™.

In this research, complete *polA* gene was cloned but the Klenow fragment activity of *Bst* DNA polymerase I was verified by LAMP. To the best of our knowledge, production of Klenow fragment of *Bst* DNA polymerase, purification, and the verification of the enzymatic activity in LAMP have not yet been reported in Iran.

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Cloning and Expression of Bst DNA Polymerase I Gene in E. coli BL21

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چکیده

مقدمه: لیپید میکروبی به علت خواص درمانی اسیدهای چرب، توجه پژوهشگران را به طور گسترده‌ای جلب کرده است. هدف این مطالعه تولید و بهینه‌سازی لیپیدها و اسیدهای چرب توسط قارچ *مورتیرلا وینشیا*، با استفاده از محیط‌های کشت مختلف بود تا اسیدهای چرب با ارزش برای استفاده در صنایع غذایی و دارویی به دست آید.

مواد و روش‌ها: قارچ *مورتیرلا وینشیا* روی محیط کشت پوتیتو دکستروز آگار کشت داده شد. سپس اسپورها به محیط تولید تلقیح داده شدند. بعد از ۷۲ ساعت، لیپیدها استخراج و توسط کروماتوگرافی گازی آنالیز شدند. برای بهینه‌سازی تولید لیپید و اسیدهای چرب مهم در محیط کشت، منابع کربنی و نیتروژنی مختلفی به ترتیب جایگزین گلوکز و عصاره مخمر شد.

نتایج: اثر چندین منبع کربن و نیتروژن بر روی بیومس، تولید لیپید و اسیدهای چرب بررسی شد. بیشترین میزان تولید لیپید در محیط کشت حاوی لاکتوز و عصاره مخمر یافت شد (۲۶/۶۶درصد). لینولئیک اسید تنها در حضور لاکتوز و عصاره مخمر (یا پپتون) تولید شد (۲۵/۷درصد). با این حال، *مورتیرلا وینشیا* بیشترین سطح لینولئیک اسید (۵۲/۷۶درصد) را در محیط کشت حاوی پپتون تولید کرد. لینولنیک اسید تنها در حضور لاکتوز و تریپتون به دست آمد.

بحث و نتیجه‌گیری: در این مطالعه، لاکتوز به عنوان منبع کربن بیشترین تأثیر را بر تولید لیپید داشت. علاوه بر این، لینولئیک اسید در حضور لاکتوز تولید شد، بنابراین لاکتوز به عنوان بهترین منبع کربن انتخاب شد. پپتون و تریپتون به عنوان منبع نیتروژن به ترتیب برای تولید لینولئیک و لینولنیک اسید توسط *مورتیرلا وینشیا* انتخاب شدند. تمام این یافته‌ها نشان می دهد که سویه مورتیرلا یک کاندید بالقوه برای افزایش محتوای لینولئیک اسید و لینولنیک اسید است. همچنین، این شرایط ساده محیط کشت می‌تواند برای تولید لینولئیک اسید و لینولنیک اسید در مقیاس بالاتر، برای اهداف صنعتی استفاده شود.

واژه‌های کلیدی: اسید چرب، استخراج لیپید، کروماتوگرافی گازی، *مورتیرلا وینشیا*

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