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Molecular characterization of thermostable DNA polymerase of *Bacillus stearothermophilus* spp isolated from soil in Bangalore, India

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ABSTRACT

Thermostable DNA polymerases are extensively used in DNA amplification reactions such as the Polymerase Chain Reaction (PCR), require the activity of the enzymes at high temperatures. The aim of the present study was to evaluate the probable biotechnological potential of Indian thermostable DNA polymerases. As a result, in the present study, DNA polymerase gene isolated from Bacillus stearothermophilus and amplified using Primer 3 plus software was ligated with T vector (pTZ57R/T) and transformed into DH5α cells. The plasmid DNA obtained, was then confirmed by restriction digestion and sequence analysis. The sequence was found to be 99% similar to a published sequence (GenBank sequence in NCBI). The study also suggests that the optimization of the enzyme activity might increase its use in PCR methods further.

Key words: *Bst Polymerase, Bacillus stearothermophilus,* Cloning, PCR

INTRODUCTION

Thermophilic enzymes, which assure principle biotechnological advantages against mesophilic enzymes, are widely used in the PCR reaction exploiting thermostable DNA polymerases [1, 2, 3]. Following the discovery and study of DNA polymerase I from E.coli in the year 1950s, many studies have isolated and characterized a continuum of DNA polymerases from both prokaryotic and eukaryotic organisms. The thermostable enzyme, which was isolated from Thermus aquaticus and characterized for the first time, is Taq DNA polymerase [4]. Other DNA polymerase enzymes which were studied and applied to PCR application were Tfl, Tth, and Tfi polymerases [5, 6, 7, 8]. However, these enzymes base insertion fidelity is low owing to the absence of 3´→ 5´ exonuclease activity. During polymerization, DNA polymerases associated with the 3´ → 5´ exonuclease dependent activity is necessary for the proof reading. A large number of DNA polymerases with the 3´ → 5´ exonuclease dependent proof reading activity were identified and used for PCR amplification [9,10].

Although *Taq* DNA polymerase and some other thermostable DNA polymerases have considerable reverse transcriptase (RT) activity, DNA polymerase from *Thermus thermophilus possess* a remarkable reverse transcriptase activity that can be employed in RT-PCR [11, 12]. As the thermostable DNA polymerase plays an important role in PCR technology, demand for various thermostable DNA for the growing applications of using PCR has increased

drastically [13]. As a result, the objectives of the study is to identify and clone the sequence of thermostable DNA polymerase I obtained from Bacillus stearothermophilus isolated from various soil environments in India.

MATERIALS AND METHODS

Isolation of thermophilic bacteria from soil and Isolation of genomic DNA

Soil samples from various locations from Bangalore were collected and 1 gram of sample was added to 20ml distilled water. The soil suspensions were serially diluted 10 fold and ~100µL for each dilution was placed on nutrient agar plates and were incubated at 65°C overnight. We were able to obtain certain bacterial growth at 65°C and the putative thermophilic bacteria which grew on the plates were further streaked on nutrient agar plates to obtain isolated colonies. The colonies were picked and sub-cultured in 10ml of nutrient broth for the isolation of genomic DNA. Total genomic DNA from the bacteria was isolated by N- Cetyl- N, N, N-trimethyl- ammonium bromide (CTAB) method. The quantity of the isolated DNA was checked in UV-VIS spectrophotomer (Vivaspec Biophotometer, Germany). From the stock 1µl DNA was mixed with 49-µl sterile distilled water to get 50 times dilution. The A260/A280 ratio was recorded to check the purity of DNA preparation.

Primer Designing and synthesis

The specific primers were designed using Primer3 Plus software (http://www.bioinformatics.nl/ cgi-bin/primer3plus/primer3plus.cgi/) and the designed oligonucleotides were synthesized in Sigma Corporation USA. PCR amplification of *bst* polymerase gene was done using the primers such as: FP- 5'TACGATTCATTCCCGAAG C3'. RP - 5' CTTTTCCATAGACCGCCTCA3'. The product size expected to be 1400 bp.

Cloning of the *Bst Polymerase* gene

The eluted PCR products were ligated separately into the pTZ57R/T cloning vector (Fermentas, USA). The amount of PCR product to be used for 1:1 vector to insert ratio for each ligation reaction. The ligated product was later kept on ice until the transformation experiment started.

The ligated product was mixed with prepared competent cells and incubated on ice for 30 minutes followed by heat shock was given to the ligation and competent cell mixture at 42°C for 2 minutes. The tubes were transferred quickly onto ice and incubated for 2-3minutes. 1ml of LB broth was added and the tubes were placed in an orbital shaker at 37°C for 1 hour with an agitation of ~200rpm. After incubation, the tubes containing cells were centrifuged at 1000rpm for 10 minutes at room temperature and resuspended the pellet in 100 µl of fresh LB broth. From the suspension, 100 µl was spread on Ampicillin containing LB agar plate using a bent sterile glass rod. The plates were incubated at 37°C overnight.

Plasmid isolation and sequencing

White coloured colonies (containing recombinant plasmids) were selected and streaked on a fresh LB plate containing ampicillin and incubated overnight and served as a master plate. Colonies were subjected to plasmid DNA isolation and restriction analysis in order to identify the positive recombinants. Plasmid isolation was done by alkaline lysis method. Followed by the purified plasmid was subjected to restriction digestion using restriction endonucleases such as *Bam* HI and *Eco* RI (Merck, India). Restriction digestion was performed in 20 µl reaction volumes with recommended units of enzyme and appropriate buffers at 37°C for 4h. The products of restriction digestion were resolved in 1% agarose gel for confirming the release of the insert by the restriction endonucleases.

Our gene insert was eluted from the Agarose gel using gel extraction kit (Bioline USA). The insert in the purified plasmid was sequenced using ABI PRISM Big Dye Terminators v1.1 cycle sequencing kit (Applied Biosystems Foster city, CA, USA) according to the manufacturer's instruction employing T7 or M13 primers. The comparison of the nucleotide sequences of the unique fragment with the sequences available in the GenBank database was carried out using the NCBI BLAST program (http//www.ncbi.nlm.nih.gov/blast).

RESULTS AND DISCUSSION

Genomic DNA isolation and quantification

The putative thermophilic bacteria were cultured in the Nutrient broth media and genomic DNA was isolated by modified CTAB method. The isolated DNA was electrophorized in 1% Agarose gel (Fig.1). The quantity and

quality of DNA was analyzed by UV visible spectrophotometer and the data was shown in the table 5. A260/280 value showing the purity of the isolated DNA.

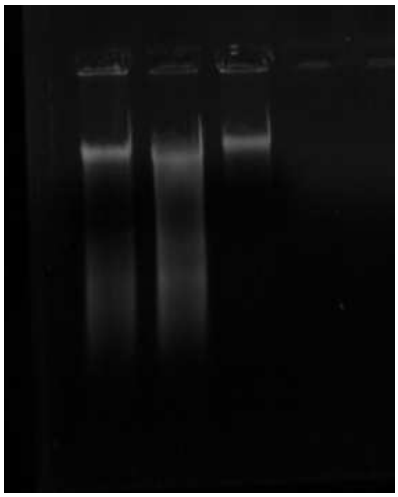


Figure 1: Genomic DNA isolated from putative Bacillus stearothermophilus

PCR amplification of the *Bst polymerase* gene

Species specific primers were designed for *Bst polymerase* gene *of Bacillus stearothermophilus* using Primer3 software. The predicted primers were validated *in silico* and subsequently in wet lab. The primers could yield an amplicon of the expected size specific to *Bst polymerase* gene. The primers were found to produce ~1400 bp amplicon which shown in the figure 2.

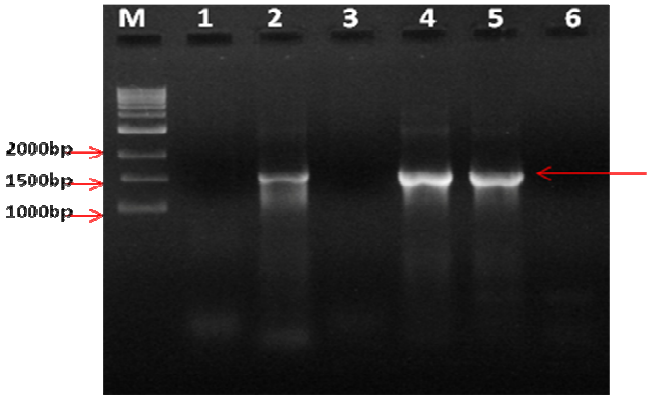
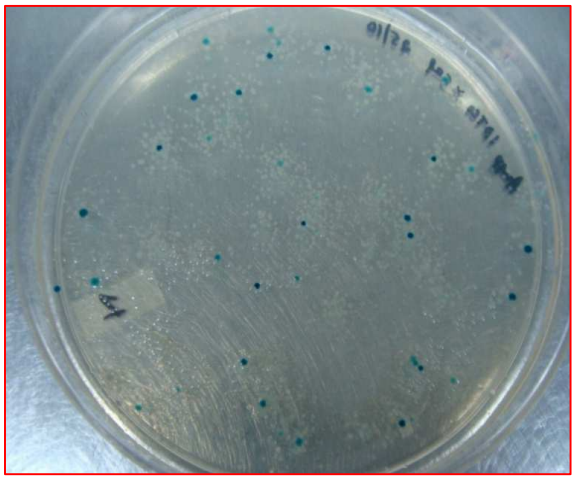


Figure. 2: PCR amplification of *Bst pol* gene by specific primers

Cloning of PCR product in to T vector

PCR yielded a specific amplicon of 1400 bp in the strain (Fig. 2). The fragment was separated in gel and purified the product using gel extraction kit. The purified PCR product was quantified and ligated with cloning vector (Fig. 3) using T4 DNA ligase enzyme.



The ligated plasmid was transformed in to *E.coli* bacterial strain DH5α. The transformation was done by heat shock method and transformed cell was cultured in the Xgal-IPTG-Ampicillin-LB Agar plate at 37^{o}C for overnight (Fig.4). The white colonies were picked up from the plates and cultured in Ampicillin containing LB broth

Plasmid Isolation

Plasmid was isolated from the transformed cells by using alkaline lysis method. The isolated plasmid was further analyzed by performing Agarose gel electrophoresis (Fig.5).

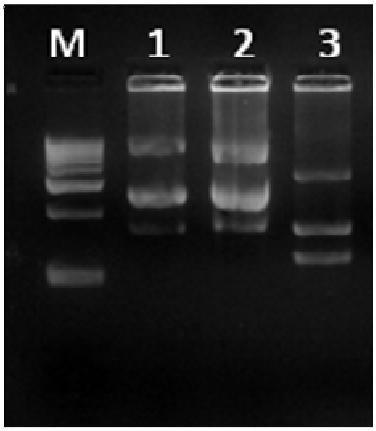


Figure.5. Plasmid isolated from the transformed bacterial cells

Sequence data

The Bst polymerase gene isolated in-house was identified by DNA sequence analysis of the isolated plasmid. The DNA sequencing performed at Eurofins, Bangalore produced a 1400 bp DNA sequence. The sequence data is shown below**.**

Bst polymerase gene sequence (in-house)

5’TACGATTCATTTCCCGAAGCCGGAGCGGTAGCCGGCTTCTTTTTATGGCCCCCGCCGGCGTGGTACA
ATAGAACAAGGAACGTCCGAGGAGGGATGATGTTGAAAAACAAGCTCGTCTTAATTGACGGCAACAG
CGTGGCGTACCGCGCCTTTTTTGCGTTGCCGCTTTTGCATAACGATAAAGGGATTCATACGAACGCAGT
CTACGGGTTTACGATGATGTTAAACAAAATTTTGGCGGAAGAGCAGCCGACCCACATTCTCGTTGCGT
TTGACGCCGGGAAAACGACGTTCCGCCATGAAACGTTCCAAGACTATAAAGGCGGGCGGCAGCAGAC
GCCGCCGGAACTGTCGGAACAGTTTCCGCTCGTGCGCGAATTGCTCAAAGCGTACCGCATCCCCGCCT
ATGAGCTCGACCATTATGAAGCGGATGACATCATCGGAACGATGGCGGCGCGGGCTGAGCGAGAAGG
GTTTGCAGTGAAAGTCATTTCCGGCGACCGCGATTTAACCCAGCTTGCTTCCCCGCAAGTGACGGTGG
AGATTACGAAAAAAGGGATTACCGACATCGAGTCGTACACGCCGGAGACGGTCGTGGAAAAATACGG

CCTCACCCCGGAGCAAATTGTCGACTTGAAAGGATTGATGGGCGACAAATCCGACAACATCCCTGGCG
TGCCCGGCATCGGGAAAAAAACAGCCGTCAAGCTGCTCAAGCAATTCGGCACGGTCGAAAACGTACT
GGCATCGATCGATGAGATCAAAGGGGAGAAGCTGAAAGAAAATTTGCGCCAATACCGGGATTTGGCG
CTTTTAAGCAAACAGCTGGCCGCTATTTGCCGCGACGCCCCGGTTGAGCTGACGCTCGATGACATTGTC
TACAAAGGAGAAGACCGGGAAAAAGTGGTCGCCTTGTTTCAGGAGCTCGGATTCCAGTCGTTTCTCGA
CAAGATGGCCGTCCAAACGGATGAAGGCGAAAAGCCGCTCGCCGGGATGGATTTTGCGATCGCCGAC
AGCGTCACGGACGAAATGCTCGCCGACAAAGCGGCCCTCGTCGTGGAGGTGGTGGGCGACAACTATC
ACCATGCCCCGATTGTCGGGATCGCCTTGGCCAACGAACGCGGGCGGTTTTTCCTGCGCCCGGAGACG
GCCGTCGCCGATCCGAAATTTCTCGCTTGGCTTGGCGATGAGACGAAGAAAAAAACGATGTTTGATTC
AAAGCGGGCGGCCGTCGCGCTAAATGGGAAAGGAATCGAACTGGCTGGCGTCGGCGTCGTGTTCGAT
CTGTTGCTGGCCGCTTACTTGCTCGATCCGGCGCAGGCGGCGGGCGACGTTGCCGCGGTGGCGAAAAT
GCATCAGTACGAGGCGGTGCGATCGGATGAGGCGGTCTATGGAAAAG3’

The DNA sequence obtained was used to investigate the identity of the putative DNA *polymerase* gene of *Bacillus stearothermophilus*. A Basic Local Alignment Search Tool BLAST (version 2.7) analysis was performed to establish the quality and accuracy of DNA sequencing results.

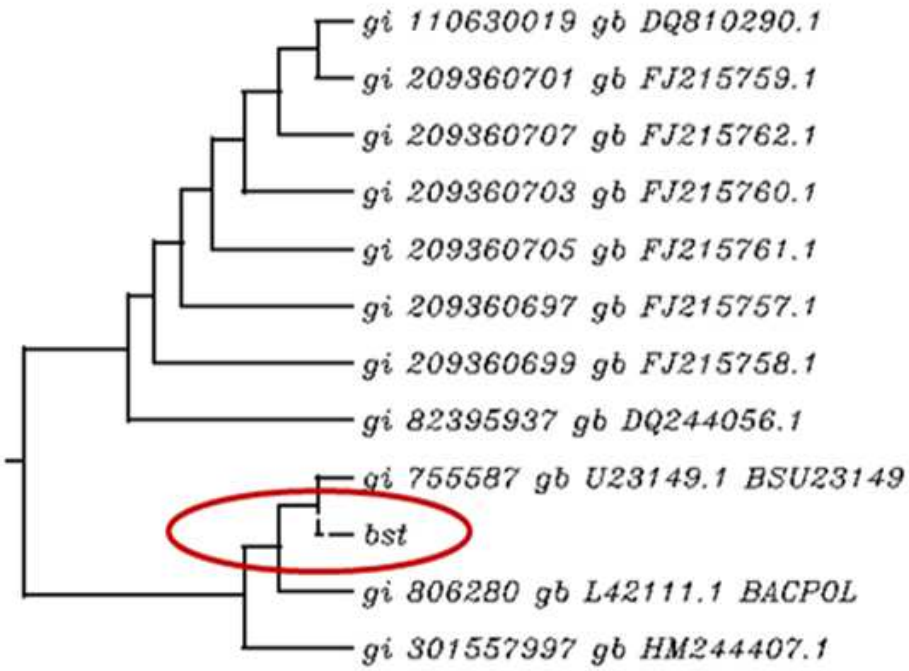


Figure.6: Phylogenic tree depicting the relationship of bst polymerase gene isolated in-house with the matching thermophilic DNA polymerases

The sequences were compared to their corresponding GenBank sequences. The in-house *bst polymerase* sequence showed 100-99% similarity with thermophilic DNA polymerase gene sequences available from GenBank database with a significant e value of 0.0 (as shown in the figure.6)

Prevailing evidence suggest [14] that there are variation in the activities of native and recombinant proteins and also, points out that these differences in general might be owing to the effects of factors like salts, particular chaperones, and other composites that are characteristics of these thermophilic bacteria [7, 13]. The present study substantiates the polymerase activity of the enzyme *Bst* DNA polymerase I qualitatively at 70 °C. The study also suggests that further quantitative analysis and optimization of the conditions would clarify the probable potential of this enzyme for use in PCR amplifications. It is also foreseen that based on the contiguous DNA polymerase sequence, that this enzyme possibly will have a obvious reverse transcriptase activity that needs to be explored.

CONCLUSION

In the current study, cloning, expression and characterization of thermostable DNA polymerase from Bacillus stearothermophilus were reported. Comparison of gene sequence from GenBank database showed that the DNA polymerase gene sequence from Bacillus stearothermophilus strain was 99% identical to that from the published sequence in NCBI. The thermal stability of the DNA polymerase along with its extensive and elevated activity between 45° C and 65° C makes it a good contender for biotechnological use.

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