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CLONING OF DNA POLYMERASE-1 GENE FROM THERMOPHILIC *Bacillus licheniformis* STRAIN NWMF1 INTO AN *E.coli* EXPRESSION SYSTEM

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ABSTRACT

DNA polymerase, catalyze template directed synthesis of DNA from nucleotide triphosphate. Thermostable DNA polymerase-I (DNAP-1) has been a common reagent in molecular biology because of its use in DNA amplification and DNA sequencing by PCR. DNAP-1 produced in moderate thermophiles such as *Bacillus* species may not be suitable for PCR, However, moderately thermophilic DNAP-1 from *Bacillus* has been used in molecular biology techniques such as loop mediated isothermal amplification. It is a low cost alternative to detect certain infectious diseases such as tuberculosis, malaria and can be applied in low/middle income countries. The objective of the study was isolation and cloning of DNAP-1 gene from native thermophilic bacterium, *Bacillus licheniformis* strain NWMF1 and over-expression by using expression host *E. coli* BL21(DE3)pLysS. A gram +ve endospore forming thermophilic bacterium was isolated from soil near the hot-water springs at Polonnaruwa, Sri Lanka. The identification of *Bacillus licheniformis* strain NWMF1 was carried out using morphological tests and 16s r.RNA gene sequence analysis. Initially the gene was cloned into pGEMT-easy vector and transformed into *E. coli* JM109 followed by sequence confirmation and protein blast analysis by NCBI. Thereafter the DNAP-1 gene re-cloned into PET28a+ vector and transformed into *E. coli* BL21(DE3)pLysS expression host. Recombinant *E. coli* clones were confirmed by colony PCR. Sequence analysis confirmed the presence of the complete gene (2640bp) including start and stop codons. The complete protein sequence consists 879 amino acids. SDS-PAGE and analysis by EXPASy–ProtParam indicated the molecular weight of DNAP-1 as ~92 kDa. Polymerase activity of His-tag purified DNAP-1 was demonstrated by PCR methodology.

Keywords: *Bacillus licheniformis*, Cloning, DNA Polymerase-1, Expression

INTRODUCTION

The thermostable DNA polymerases, like other DNA polymerases (E.C 2.7.7.7), catalyze template directed synthesis of DNA from nucleotide triphosphates. Commercial preparations of DNA polymerases have a variety of applications in DNA manipulations *in vitro*, such as sequencing, labeling, cDNA synthesis etc. (**Ishino and Ishino, 2014**).These enzymes are commercially produced in convenient, high-yielding mesophilic hosts such as *E. coli*, by the use of recombinant DNA technology. DNA polymerases such as *Taq* polymerase which have an optimal temperature of ~80°C are extensively used in PCR. DNA polymerases from moderate thermophiles have uses in molecular diagnostic techniques such as Loop Mediated Isothermal Amplification (LAMP). It is a low cost alternative to PCR that can be applied in low and middle-income countries for screening /diagnosis of infectious diseases (**Mori *et al.*, 2013**). LAMP has been observed to be less sensitive to inhibitors in clinical samples when compared to PCR and successful detection of pathogens from minimally processed samples such as heat-treated blood has been reported (**Curtis *et al.*, 2008: Sattabongkot *et al.*, 2014**). Further, they can be applied for *in vitro* DNA manipulation techniques in the molecular biology, where a higher temperature reaction is more suitable.The objectives of this project were to clone and over-express the DNA polymerase I (DNAP-1) gene from a thermophilic bacterium, with a view to subsequent scale up.

MATERIALS AND METHODS

The *Bacillus licheniformis* strain NWMF1 was cultured in LB medium at 55°C with constant shaking. Plasmid DNA isolation was by alkaline SDS method (**Sambrook *et al.*, 2012**). DNA fragments were purified from agarose gels using Wizard SV gel purification system (Promega USA). All DNA ligations were carried out using Liga-Fast Rapid DNA Ligation System according to manufacturers' protocol. Sanger sequencing was outsourced (Macrogen, Korea). DNA and translated amino acid sequences were analyzed by nucleotide and protein BLAST tools using online sequence databases available at NCBI.

Isolation and identification of thermophilic bacteria

The thermophilic bacterium was isolated from soil near a hot water spring at Nelumwewa, Polonnaruwa District, Sri Lanka, by enrichment for growth at high temperature (~60°C). The isolated bacterium was characterized morphologically and biochemically (Grams reaction and catalase test). Molecular identification was based on 16s RNA gene sequencing (using 27F and 534R universal primer pair). The 16s rRNA analysis and biochemical characteristics of bacteria were compared to confirm the bacterial species as *Bacillus licheniformis* strain NWMF1

Cloning of *B. licheniformis* DNA polymerase-1 gene

Multiple sequence alignment of open reading frames (ORF) of *B. licheniformis* strain NWMF1, DNAP-1 genes were carried out to identify conserved sequences for designing of the PCR primers. NcoI and BamHI restriction enzyme recognition sequences were added to the 3' region of the forward and reverse primers respectively. Further, the His-tag purification sequence $(CAC)_6$ was added to the reverse primer. The Ompa (Outer Membrane Protein-A) signal sequence was added to the forward primer. The forward primer, DNAP-1F (5'-GCA TGA CCA TG GGT ATG AAA AAG ACA GCT ATC GCG ATT GCA GTG GCA CTG GCT GGT TTC GCT ACC GTT GCG CAA GCT ATG ACT GAA AAA AAA TTA GTA TT-3') and the reverse primer DNAP-1R (5'-GCA TGA GGA TCC CTA CAC CACCACCACCACCAC TTT TGC ATC GTA CCA TGAA-3') were custom-synthesized.

Bacterial genomic DNA was isolated according to the method of **Dubnau (1982)**. The isolated *B. licheniformis* strain NWMF1 was PCR- amplified from genomic DNA, gel-purified and cloned into PGEM^R-T easy vector (Promega) and transformed into *E.coli* JM109 high efficiency competent cells. Recombinant plasmid DNA was isolated and confirmed by sequencing.

The recombinant plasmid was digested with Nco1 and BamHI restriction enzymes to remove the cloned the DNAP-1 gene and subsequently gel-purified. It was then cloned into the NcoI/BamHI site of pET 28a+ expression vector and transformed into *E. coli* BL-21(DE3)pLysS high efficient competent cells, compatible with kanamycin selection. Selected recombinants were screened by

colony PCR and confirmed by sequence analysis as previously stated. Additionally, Interpro (EMBL-EBI) protein analyses were performed to detect conserved domains in the translated protein sequence. Structural and functional features of the protein were obtained from Predictprotein online prediction tool, UniProt protein database and Protein Data Bank (PDB).

Purification and analysis of expressed recombinant DNAP-1 protein

Extracellular DNA Polymerase-I production from recombinant *E. coli* BL-21(DE3)pLysS cells were induced by IPTG. The supernatant of the LB broth was separated and over expressed DNA Polymerase-I were purified by using MagneHis protein purification system (Promega, USA) according to the manufacturer's instructions.

The activity of recombinant, purified DNAP-1 was demonstrated by using a modified protocol of a previously optimized 30-cycle PCR program to amplify the alkaline protease gene from *B. licheniformis* (**Wanigasekara *et al*., 2016**). In this protocol, the extension step was carried out at 62°C and1µl of enzyme (out of a total of 60 µl of His-tag purified enzyme extract) was added in each cycle at the primer annealing step, in order to accommodate the moderate thermostability of the recombinant DNAP-1.

Protein concentration was measured by the bicinchoninic acid assay (**Smith *et al*., 1985**) with BSA as the standard. Protein samples were prepared for SDS-PAGE as described in **Tang *et al*., (2001)** and analyzed according to the method of **Laemmli (1970)**.

RESULTS AND DISCUSSION

The selected bacterium showed optimum growth at 55°C. The Gram positive, motile, catalase positive, spore forming, rod shaped bacterium was identified as *Bacillus licheniformis* and the strain was designated as NWMF-1.

The PCR amplified DNAP-1 gene from *B. licheniformis* NWMF-1 was observed between 2500 bp and 3000 bp (Figure 1).

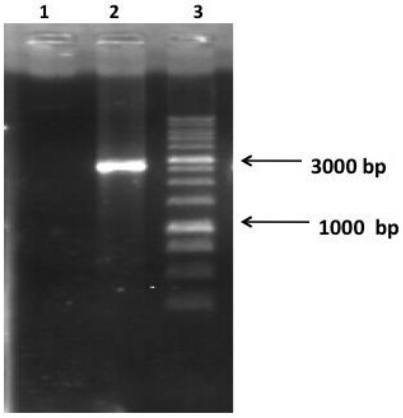

Figure 1 Gel electrophoresis photographs of PCR amplified DNAP-1 gene from *Bacillus licheniformis* strain NWMF1. Lane 1: Negative control, Lane 2: PCR amplified DNAP-1 gene, Lane 3: 1kb DNA ladder (Promega).

Sequence analysis revealed that the complete coding sequence was 2640bp. The sequence has been submitted to the NCBI data base and is available under the accession number: MF536412.1. Nucleotide BLAST (NCBI) of the complete nucleotide sequence obtained for *B. licheniformis* NWMF-1 DNAP-1 gene showed 99% identity with DNA polymerase-I gene from *B. licheniformis* DSM13 = ATCC 14580 (AE017333.1) and *B. licheniformis* BL1202 (CP017247.1).

PCR-amplification of the alkaline protease gene (1140 bp) from *B. licheniformis* (NCBI data base accession number: MF496035.1) using His-tag purified recombinant DNAP-1 revealed a DNA fragment of expected size during electrophoresis on a 0.8% agarose gel. This indicated that the recombinant DNAP-1 enzyme was active (Figure 2).

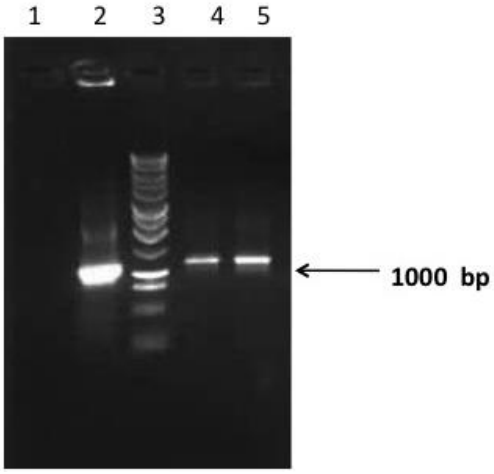

Figure 2 Gel photograph of PCR-amplified alkaline protease gene using recombinant DNAP-1 enzyme. Lane 1: negative control (No enzyme), Lane 2: positive control (amplified using GoTaq polymerase), Lane 3: 1kb ladder, Lanes 4 and 5: amplified products using recombinant DNAP-1 enzyme.

SDS-PAGE of His-tag purified recombinant DNAP-1 revealed a single protein band between 75-100kDa (Figure 3). The size of the protein was approximately 92 KDa, as obtained by the EXPASy – ProtParam tool.

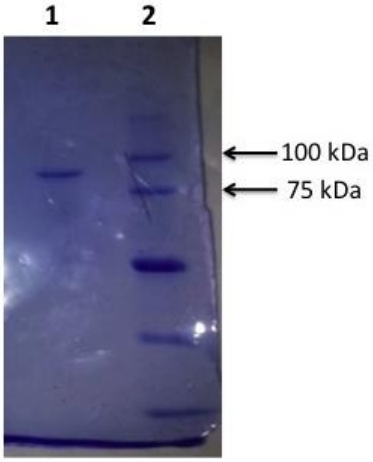

Figure 3 SDS PAGE gel photograph for His-tag purified DNAP-1. Lane 1: DNAP-1. Lane 2: Broad ranged protein ladder.

The complete amino acid sequence obtained for *B. licheniformis* NWMF-1 DNAP-1 consisted of 879 amino acids and it showed 100% identity with *Bacillus licheniformis* strain NWMF1 DNA polymerase-I protein sequences from UniProt (EMBL-EBI).

Structural features of the DNAP-1 was obtained using ProteinPredict online tool (Yachdav*et al*, 2014). The active site of the mature peptide contains 24 amino acid residues (aspartic acid; positions 11, 61, 112, 113, 136, 138, 833, histidine; positions 685, 832, serine; position 620, threonine; positions 614, 616, 793, glutamate; position 110, arginine; position 618, 705, 792, glutamine; position 615, 800, leucine; position 619, valine; position 831, lysine; position 709, tyrosine; position 717 and asparagine; position 796). The conserved 5' to 3' exonuclease domain (PIN_53EXO) of family A DNA polymerases was also observed in *B.licheniformis* NWMF-1 DNAP-1. The predicted secondary structure consists of 36.2% loop, 55.4% helix and 8.4% strand (**Rost *et al*, 2004**). The predicted protein contains a single disulphide bond.

CONCLUSION

A moderately thermostable bacterium isolated from soil near a hot water spring was identified as *Bacillus licheniformis.* The strain was designated as NWMF-1. The DNA polymerase-I gene from *Bacillus licheniformis* strain NWMF1 was over expressed in an *E. coli* expression system. The His-tag purified DNAP-1 showed polymerase activity while carrying the histidine tag and is expected to improve when the tag is cleaved. The cloned DNAP-1 has potential for application in loop mediated isothermal amplification (LAMP) and other molecular biology techniques, especially in developing countries.

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