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Cloning of Thermostable *DNA Polymerase* Gene from a Thermophilic *Brevibacillus sp.* Isolated from Sikidang Crater, Dieng Plateu, Central Java

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Abstract

Thermostable DNA polymerase has an important role for amplifying small amount of DNA through polymerase chain reaction (PCR). Thermophillic bacteria *Brevibacillus sp.* was isolated from Sikidang Crater, Dieng Plateu, Central Java. Previous study showed that crude protein of the isolate could be used in PCR. Unfortunately, like most native thermostable enzymes, the thermostable DNA polymerase of the isolate is synthesized in a very low level and therefore is cumbersome to purify. The purpose of this research is to clone thermostable DNA polymerase gene of the isolate. The DNA polymerase gene was amplified by means of PCR using spesific primers. The amplified fragment was then isolated, purified, and ligated into the pGEM-T cloning vector. The recombinant plasmid was then transformed to competent *E. coli* JM109 cells using heat shock method. The cloned thermostable DNA polymerase gene from the thermophilic isolate was then characterized for its nucleotide base sequence.

The result showed that the DNA *Pol I* gene was successfully be amplified from the isolate DNA genom, resulting in ± 2,7 kb DNA fragment in length. Sequence analysis of segment of targeted gene showed high similarity to that of thermostable DNA polymerase genes from other *Bacillus*.

Key words : Thermostable DNA *Pol I*, *Brevibacillus sp*., PCR, cloning

Introduction

Hyperthermophilic and thermophilic bacteria from geothermal sites have some thermostable enzymes which enable them to survive in high temperature. Thermostable DNA Polymerase I (DNA *Pol I*) is important enzyme in molecular biology research, particularly for the amplification of a small amount DNA. Several species of genus *Bacillus* were reported as hyperthermophilic bacteria which express thermostable enzymes (Allan *et al*., 2005). The *Bacillus* DNA *Pol I* was isolated and cloned from *Bacillus caldotenax* (Uemori *et al*., 1993), *Bacillus stearothermophilus* (Phang *et al*., 1995), *Geobacillus sp. MKK* (Khalaj-Kondori *et al*., 2007), *Geobacillus thermoleovorans* (Akhmaloka *et al*., 2008) and *Geobacillus caldoxylosilyticus* TK4 (Sandalli *et al,* 2009) and it has been biochemically characterized.

Several thermophilic bacteria from Sikidang crater, Dieng Plateau, Central Java were previously isolated (Ardiansyah, 2006). An isolate known as *Brevibacillus sp.* could grow well at 50-70?°C. Crude protein of the isolate cell extract was able to amplify a small amount of DNA template in PCR (Wibawa, 2008). This result indicated that crude protein of the isolate contains a thermostable DNA polymerase. There was still no published

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report regarding a thermostable DNA polymerase from *Brevibacillus sp*. Like most native thermostable enzymes, the thermostable DNA polymerase of the isolate is synthesized in a very low level and therefore is cumbersome to isolate and purify.

The objective of this research was to clone a gene encoding thermostable DNA polymerase from the previously isolated *Brevibacillus sp.* using PCR. Encoding DNA polymerase gene of the isolate will be amplified by PCR using spesific primers. The amplified fragment was then isolated, purified, and ligated into pGEM-T cloning vector. The recombinant plasmid was then used to transform competent *E. coli* JM109 cells using heat shock method. The cloned gene was then characterized for its nucleotide base sequence.

Materials and Methods

Bacterial strains and plasmids

Brevibacillus sp. was isolated from soil sample taken from Sikidang crater Dieng Plateau, Central Java (Ardiansyah, 2006). The strain used for cloning was *E. coli* JM109 (*recA1, endA1, gyrA96, thi, hsdr17* ($r_{k-}m_{k+}$), *relA1, supE44, D(lac-proAB), [F', traD36, proAB, laclqZDM15*]). The plasmid used for cloning vector was pGEM-TÒ (Promega).

Growth condition

Brevibacillus sp. was grown at 70^0C in modified LB (Luria Bertani) broth which contain 1% Bacto Tryptone, 0.5 % yeast extract, 0.5% Mannitol, 0.02 NaCl, 0.01% K_2HPO_4, 0.02% $CaCl_2$, 0.01% $MgSO_4.7H_2O$, and 0.01% $FeCl_2$. *E. coli* JM109 strain was grown at 37^0C in LB broth or plated on LB agar containing the appropriate antibiotics when required.

Bacterial growth assay

A single colony of bacteria culture was inoculated into 100 ml modified LB broth then incubated overnight at 70°C in a waterbath with vigorous shaking. A hundred microliter cultures were inoculated into 5 ml modified LB broth. These cultures were incubated at 50°C, 60°C, 70°C and 80°C on waterbath shaker.

Brevibacillus sp. DNA genome isolation

Cells grown in modified LB broth were harvested and washed using 100 µl of TEN buffer (Tris-Cl 10 mM pH 7,9; EDTA 1 mM; NaCl 1 M). Then the pellet was resuspended with 200 µl of lysis solution (Tris-Cl 10 mM pH 7,9; EDTA 1 mM; NaCl 1 M and Lisozyme 1 mg/ml) then was incubated at 37°C for 10 min. The cell wall was disrupted by adding 225 µl TEN-Sarkocyl buffer (Tris-Cl 10 mM pH 7,9; EDTA 1 mM; NaCl 1 M and 2% sarkocyl) then incubated at 42°C for 1 h. Proteinase K (0,1 mg/ml) was added and then incubated at 42°C overnight. DNA genome was purified using phenol:chloroform extraction method. All subsequent steps were molecular biology standard according to Sambrook *et al.* (2001).

Preparation of primers and PCR

The internal primers were designed based on the conserved region in family A DNA polymerases (Uemori *et al.*, 1993). The forward primer (PolI-Fint) was 5'-GAY CCH AAC YTS CAR AAY ATH CC-3' and the reverse primer was (PolI-Rint) 5'-KAS SAK YTC RTC GTG NAC YTG-3'. The external primers were designed based on the region of the product gene which amplified using internal primer. The PCR was performed at 94°C for 5 min (initial denaturation) and followed by 25 cycles of 94°C for 45 sec (denaturation), 49°C and 44°C for internal and external primers, respectively at 45 sec (annealing), and 72°C for 1 min (extention). After the final cycle, the PCR reaction was terminated at 72°C for 7 min. The mixture contained 1 ball of Pure Taq- Ready To Go PCR Mix (GE Health CareÒ), 40 pmol of each primer and 0,5 mg of *Brevibacillus sp.* genomic DNA.

Cloning of Brevibacillus sp. DNA Pol I into pGEM-T cloning vector

JM109 competent cells preparation

JM109 competent cells were prepared according to Inoue method (Ultra Competent cells) (Sambrook *et al.*, 2001).

Ligation

The gene amplified using external primer was cloned into pGEM-T cloning vector (Promega) using standard method.

Transformation

The ligation mixture was transformed to JM109 competent cells using heat shock method (Sambrook *et al.*, 2001). A hundred microliter of each transformation culture was then plated on LB agar containing 100 µg/ml ampicillin, 100 µl of 100 mM IPTG and 20 µl of 50 mg/ml X-Gal and incubated at 37°C overnight. Incubation was continued at 4°C overnight to facilitate blue color development.

Plasmid isolation

The obtained white colony was then isolated its recombinant plasmid using NucleoSpin® Plasmid method (Macherey-Nagel).

Plasmid digestion using restriction enzyme

Restriction enzyme, *NcoI* (Fermentas) was used for the digestion of these plasmids. The enzymatic reaction mixture was 20 µl of recombinant plasmid, 1,5 µl enzyme of 10 U/ml *Nco I*, 3 ml of 10x Tango buffer, and 5,5 µl Nuclease Free Water. This mixture was then incubated for 3 h at 37 °C.

DNA sequence analysis

Sequence of PCR products were examined at Charoen Pokhpand Indonesia, using a Dye Terminator Cycle Sequencing Ready Mix (Applied Biosystem, Foster City, USA) on PCR System 2400 and ABI PRISM 310 Genetic Analyzer (Aplied Biosystem, Foster City, USA).

Computer analysis

Primer sequence analyses were performed using Primer-BLAST program at the NCBI server and NetPrimer software. The BLAST program at the NCBI server was used for sequence similarity. Phylogenetic tree for sequence was created using BLAST Tree View program on NCBI and Clustal X software.

Results and Discussion

Bacterial growth assay

The maximum growth rate of *Brevibacillus sp.* was reached at 70°C. When the growth temperature was increased to 80°C, there was a rapid decrease in absorbance. When incubated at temperature below 50°C, there was no bacterial growth appeared. In summary, the isolated *Brevibacillus sp.* was found to grow at temperature ranging from 50-70°C. Consequently, the isolated *Brevibacillus sp.* could be classified as a thermophilic bacterium.

Amplification of DNA Pol I gene from Brevibacillus sp. DNA genome

Two steps of PCR strategy was used to amplify the whole coding sequences of DNA Pol I from *Brevibacillus sp*. genome. The first step was designed to amplify conserve region of family A DNA polymerase using internal primers. The second step was done based on sequences result from first step. The second step used external primers to amplify the whole coding region of DNA *Pol I* (Figure 1).

By using internal primers and genomic DNA from the isolated *Brevibacillus sp,* a PCR product of approximately 600 bp was obtained (Figure 2). This fragment was similar in size to the fragment of 5′→3′ polymerase domain *Bacillus caldotenax* when amplified using the same primers (Uemori *et al.*, 1993).

The fragment of polymerase domain of isolated *Brevibacillus sp.* was sequenced (Figure 3).

The sequence of the DNA fragment encoding polymerase domain of the isolated *Brevibacillus sp.* sequence was found to be homologous to DNA polymerases gene from

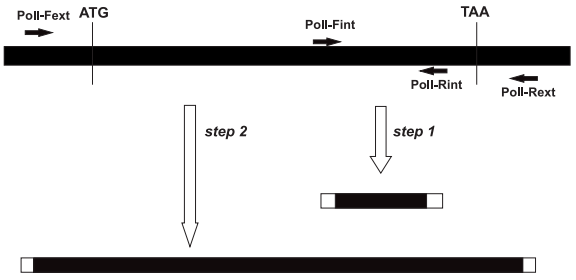

Figure 1. Cloning of DNA *Pol I* gene using two steps PCR strategy**.** The first step was done using internal primers (PolI-Fint and PolI-Rint), while the second step was carried out using external primers (PolI-Fext and PolI-Rext).

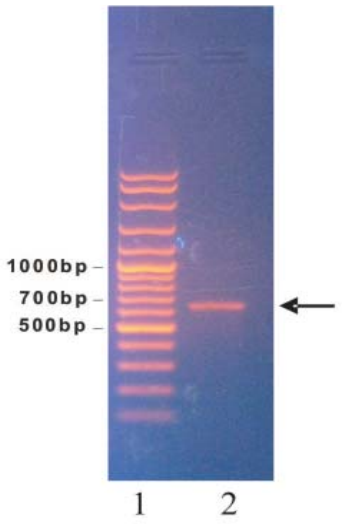

Figure 2. DNA amplification using internal primer PolI-Fint and PolI-Rint**.**
Lane 1, 100 bp DNA marker (Vivantis ®); lane 2, *Brevibacillus sp*. DNA Pol I gene

GATCCCTAACTTGCTAGAACATCCCGATTCGGCTTGAGGAAGGACG
PolI-Fint
GAAAATCCGCCCAAGCGTTCGTGCCGTCGGAGTCTGATGGCTCTATT
TTCGCTGCCGACTACTCGCCAAATGGGGTCGCGCGCCCCCGCCCTAT
TTGCGGAAGATAACCATTTAATTGGAAGCTTTCCGCCGCGTTTTGGA
TATCCCTACGAAAACAGCGATAGGACATTTTGCCAGTGAGCGAGGA
CGAAGTAGACGCCCAACATAGCGCCGTCCAGGCGAAGGCGGTCAAC
TTTAGGGATCGTTTACGGGATCCAGTAGATTACGGCTTAGGCGCAAA
ACTTAAATATTTCCACGCAAAGAGGCCGCTAGAATTCCTTCGAGCGC
TACTTCCGAAAGCTTCCCTGGCGTCGAAGCGGTATATAGGAAAACA
TTGTAGCAAGAAGCAAAACAGAAAGGGTATGTGACGACGCTGCTGC
ATCGGCGCCGCTATTTGCCGGATATAACGAGCCGCAACTTTCACGTT
CCGCAGCTTTCGCTGAACGGATAGGCGATGAACACGCCGATTCAAG
GGAGCGCCGCTGACTTATTAAAAAGGCGATGATCGATCTGAACGCC
AGACTGAAGGAAGAGCGGCTGCAAGCGCGCCTTTTTGCTGCAGGTC
CACGACGATATGGTA
PolI-Rint

Figure 3. The fragment of 5′→ 3′ polymerase domain of Brevibacillus sp. sequence. The forward primer (PolI-Fint) and reverse primer (PolI-Rint) were underlined.

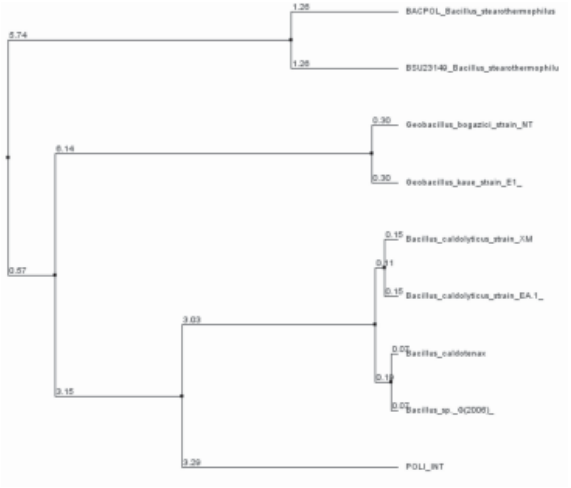

Figure 4. Phylogenetic tree for *Pol I* internal sequence using Clustal X. Calculate Tree using average distance using % identity

other *Bacillales* ordo (Figure 4). The result of comparison were shown highest similarity with DNA Pol I of *Bacillus caldotenax* (Bca). It shows 92 % match identity to *Bacillus caldotenax* DNA polymerase, *Bacillus caldolyticus* strain XM DNA polymerase, *Bacillus sp.* G (2006) DNA polymerase, and *Bacillus caldolyticus* strain EA. 1 DNA polymerase.

The second step of this research was carried out using degenerate external primers which designed based on nucleotide sequences of Bca *Pol I* gene. Both primers were designed using Primer-BLAST program on NCBI. The resulted external primers were PolI-Fext (5′-YCGAGGAGGGATGAGATTG-3′) and PolI-Rext (5′-TTATTTSGCRTCRTACCAYG-3′). By using PolI-Fext and PolI-Rext as degenerate primer, the coding sequence of *Brevibacillus sp.* DNA *Pol I* gene was obtained of approximately 2700 bp in length (Figure 5A). The result of PCR product was nearly identical to DNA *Pol I* gene from another *Bacillus* species. The DNA *Pol I* gene from *Geobacillus caldoxylosilyticus* TK4, *Bacillus*

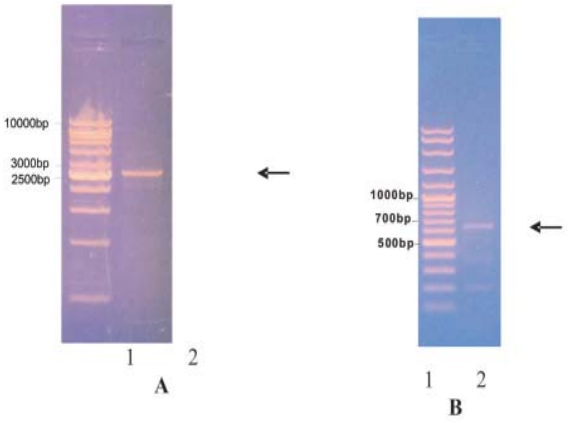

Figure 5. A. *Brevibacillus sp.* DNA *Pol I* gene amplification using PolI-Fext and PolI-Rext.
Lane 1, 1 kb DNA marker (Vivantis ®); lane 2, Brevibacillus sp. DNA Pol I gene
B. Amplification of Brevibacillus sp. *DNA Pol I* gene using internal primer.
Lane 1, the fragment of 5′→ 3′ polymerase domain; lane 2, 100 bp marker DNA (Vivantis ®)

caldotenax, Bacillus caldolyticus strain XM, Bacillus sp. G(2006), and *Bacillus caldolyticus strain EA.1* were found to be 2634 bp long, 2633 bp, 2637 bp, 2631 bp, and 2699 bp, respectively.

To verify that the isolated PCR product was *Bacillus* DNA *Pol I* gene, the isolated PCR product was re-amplified using internal primer (PolI-Fint and PolI-Rint). By using these internal primers, approximately 600 bp of PCR product was obtained, same as the fragment of 5′→3′ polymerase domain in family A DNA polymerases (Figure 5B).

The *Brevibacillus sp.* DNA *Pol I* gene (±2700 bp) was also sequenced using reverse primer 5′-CTTCGTCCTCGCTCACT-3′ (Figure 6).

This primer was designed from the fragment of 5′→3′ polymerase domain of *Brevibacillus sp.* sequence. The sequence was homologous to *Bacillus* DNA *Pol I* gene from other bacteria (Table 1).

Cloning of Brevibacillus sp. Pol I gene into pGEM-T cloning vector

An amplified product corresponding to *Brevibacillus sp.* DNA Pol I gene was cloned into pGEM-T cloning vector. The schematic diagram of constructing the plasmid recombinant was presented in Figure 7.

In this research, the presence of the insert within the plasmid was confirmed by restriction enzyme digestion. As shown as Figure 8, *Nco I* enzyme digestion produced two DNA bands. The upper band was ±5680 bp band represent the pGEMT plasmid and inserted DNA Pol I gene. The other band that was appeared slight at ±2840 bp indicated a restriction enzyme digestion by *Nco I* inside the fragment of DNA *Pol I*.

As a conclusion, DNA polymerase I gene has been successfully amplified from Brevibacillus sp. DNA genom using degenerate primer (PolI-Fext and PolI-Rext), resulting ± 2,7 kb sequence in length. Sequence analysis of clone gene showed high similarity to thermostable Bacillus DNA polymerase gene from that of other bacteria. Recombinant plasmid of Brevibacillus sp. DNA polymerase I gene and pGEM-T vector was successfully cloned into *E. coli* JM 109**.**

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ATGGATATCCAATCGCGGCGGAACGCTTCCATTAAATTGTCATCTTC
CGCAATATGGGCGAGGACGCGCAACTCAATTTGCGAGTAGTCGGCA
GCGAAAATGAGCCAATCAGACTCCGACGGCACGAACGCTTGGCGGA
TTTTCCGTCCTTCCTCAAGCCGAATCGGAATGTTTTGCAAGCTCGGC
TCCGTCGAGCTGAGCCGTCCGGTTTGCGTCAACGCCTGATTGAAAAT
CGTATGCACCTTCTTTGTATCGGGTCGCACGACTTTCAGCAAT
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Figure 6. A segment of external DNA *Pol I* sequence

Table 1. Homology analysis of a segment of external DNA sequence.

Accession	Description	ΔMax score	Total score	Query cover age	E Value	Max ident
EF488810.1	Bacillus caldolyticus strain XM DNA polymerase gene, complete cds	499	499	100%	3e-138	99%
AY247636.1	Bacillus caldolyticus strain EA.1 DNA polymerase I (polA) gene, complete cds	499	499	100%	3e-138	99%
D12982.1	Bacillus caldotenax pol gene for DNA polymerase, complete cds	499	499	100%	3e-138	99%
EF198253.1	Bacillus sp. G(2006) DNA polymerase I gene, complete cds	494	494	100%	1e-136	98%
BA000043.1	Geobacillus kaustophilus HTA426 DNA, complete genome	488	488	100%	6e-135	98%
L42111.1	Bacillus stearothermophilus DNA polymerase I (pol) gene, complete cds	296	296	99%	4e-77	86%
U23149.1	Bacillus stearothermophilus DNA polymerase (BstpolI) gene, complete cds	296	296	99%	4e-77	86%

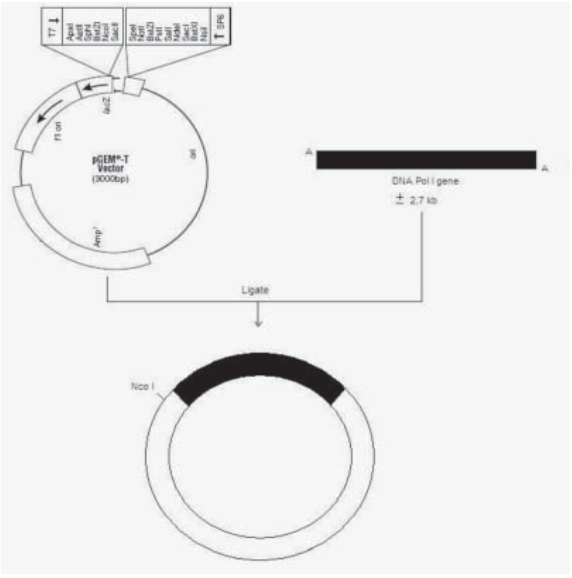

Figure 7. Construction of the recombinant plasmid DNA *Pol I*.

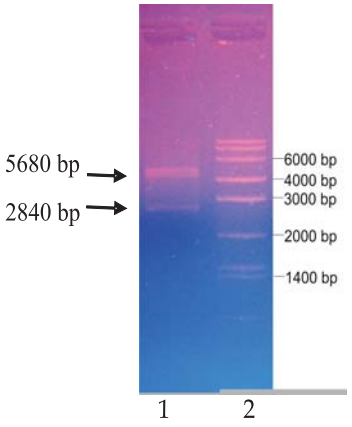

Figure 8. Structure confirmation of recombinant plasmid. Plasmid was digested with restriction enzymes Nco I.
Lane 1, Nco I digestion, lane 2, 1kb marker DNA (Sigma)

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