

In Silico Analysis for Detection of CryII Gene from Local Isolates of *Bacillus thuringiensis*

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Abstract: *Bacillus thuringiensis* (Bt) is a gram-positive bacteria that can produce crystal proteins and exhibit a high insecticidal activity. One of the cry proteins is cryII gene that encoding an insecticidal protein against the Lepidoptera insect, especially in the *Helicoverpa armigera* species, which is a corn borer. This study aimed to obtain a suitable primer candidate to amplify and characterize cryII genes from *Bacillus thuringiensis* local isolates. In silico analysis was carried out for the purpose of amplification cryII gene, using several software such as BLAST to search sequence cryII gene, Bioedit for sequence alignment, SnapGene for analysis of genes within the genome annotation, and Primer3Plus for online primer design via <https://primer3plus.com>. A pair primer was selected to represent of cryII gene with a forward primer (cry2F) 5'- ATCTGGTCTCATAGGGCGA-3' and reverse primer (cry2R) 5'-CGAGCTGTCGTGTTGCTTTG-3' with GC content percentage 55%. The purity of DNA *Bacillus thuringiensis* isolates B327 and B432 using nanoquant with a mark on each isolate at 1.88 and 1.99. Detection of the cryII gene from local isolates of Bt strain B327 and B432 were successfully amplified by Polymerase Chain Reaction (PCR) gradient technique using annealing temperatures at 50 and 54°C, with DNA band size of ±500 bp.

1 INTRODUCTION

Businesses carried out in pest control in agriculture are using natural biopesticides. Natural biopesticides are widely used by farmers because they contain *Bacillus thuringiensis* bacteria. This bacterium can be isolated from soil, water, leaf surface and various insect carcasses (Bahagawati et al., 2009). The advantages of using this bacterium as a biopesticide include its capability to be used as a specific insect repellent, due to the production of a protein called cry protein – a short for crystal protein – produced by *Bacillus thuringiensis*. The gram-positive bacterium can produce crystal proteins when sporulating in the stationary phase of its growth cycle (Jain et al., 2017). This protein contains δ -endotoxins, which are toxic to insects and nematodes, especially in the insect groups Lepidoptera, Coleoptera and Diptera (Sahin et al., 2018).

One example of the cry proteins is the cryII gene that encodes an insecticidal protein against the Lepidoptera insect, especially in the *Helicoverpa armigera* species which is a corn borer (Lone et al., 2017). Despite its toxicity to certain types of insects

(Lepidoptera, Coleoptera and Diptera), cry proteins are not toxic to many other types of insects or other organisms. When entering into digestion, δ -endotoxin is still in the form of large molecules of inactive protoxin, which will then become active in an alkaline environment (Bahagawati et al., 2009). These crystals are only protoxins, which, if dissolved in the gut of insects, will turn into shorter poly-peptides (27-149 kd) and have insecticidal properties. These toxins actively interact with epithelium cells in the eyes of insects. The toxin produced by *Bacillus thuringiensis* causes the formation of pores (very small holes) in the cell membrane of the digestive tract and disrupts the osmotic balance of the cells. Because osmotic balance is disturbed, cells become swollen and burst, causing the death of the insects (Mafazah and Zulaika, 2017).

Bioinformatics analysis based on software aims to detect genes that encode cryII proteins as biopesticides for borer pests by using specific pieces of short DNA sequences as good primary candidates for amplifying these genes. Several sophisticated methods have been developed to detect cry gene, one of them is the polymerase chain reaction (PCR)

method. PCR analysis using specific primers is the right choice to detect the presence of cryII gene sequences in genomic DNA. Optimization of the annealing temperature at the PCR stage is very important to avoid primary attachment errors. The specificity, efficiency, and sensitivity of gene amplification by PCR are influenced by several factors. One of the influencing factors is the annealing temperature (Yang et al., 2013). The aim of this study was to obtain a suitable primer candidate to amplify and characterize cryII genes. In silico analysis for the purpose amplification cryII gene produced by local isolates of *Bacillus thuringiensis* (Bt).

2 METHODS

2.1 Search for Cry II Gene on the NCBI

Search for cryII gene sequences was obtained from Genbank search results on their official site (www.nlm.nih.gov). Alignment of cryII gene sequences with closest relatives using BLAST (Basic Local Alignment Search Tools). Sequences alignment using ClustalW in the Bioedit software (v7.0.9 Tom Hall).

2.2 Primer Design and PCR in Silico

Primer design analysis using Primer3Plus software. Primers of gene sequences that have high similarity (in the base region of the cds domain) with a unique base to make the primer design and perform in silico PCR to determine the primer attachment area of the DNA template using the SnapGene software.

2.3 Pre Culture of Bacillus Thuringiensis Local Isolates

Bacillus thuringiensis local isolates were carried out by culture on liquid media Luria Bertani (LB). Bacterial cultivation by inoculating one colony from solid media to LB liquid media with a volume of 15 ml, then incubated at 37 °C into a shaker incubator at 125 rpm for 1 - 2 days. Well-grown isolates will be harvested for DNA isolation.

2.4 Preparation of Genomic DNA

The Genomic DNA was prepared from *Bacillus thuringiensis* local isolates (B432 and B327 Isolates)

cells harvested from a 1.5 mL culture follows the standard procedure of Presto mini gDNA Bacteria Kit (Geneaid).

2.5 Optimization of Annealing Temperature

Optimization of cryII gene primer annealing temperature was carried out on the DNA and the analysis was performed using Thermo Fisher software. PCR amplification was performed as many as 35 cycles using Gene Amp® PCR System 9700 (Applied Biosystem) with a gradient PCR technique to obtain optimal annealing temperature. PCR conditions were set to carry out a pre-PCR for 3 minutes at a temperature of 94 °C, followed by denaturation of 94 °C for 30 seconds. For annealing temperature optimization using a temperature range of 45 °C - 60 °C for 30 seconds, elongation at 72 °C for 60 seconds by 30 cycles. The post-PCR cycle was performed at 72 °C for 7 minutes. The PCR results were migrated in 1% agarose gel under 100-volt 40-minute conditions.

3 RESULT AND DISCUSSION

3.1 Search for CryII Gene on the NCBI

In designing the primer, one cryII gene sequence was taken as a template with an access number (AF047038.1) on the NCBI website (www.nlm.nih.gov). The cry II gene search results in GenBank got ten cry II gene candidates with 99-100% similarity, each with accession numbers (AF047038.1), (AF433645.1), (KX243304.1), (MH475905.1), (MG983754.1), (MG983753.1), (DQ064596.1), (AF273218.1), (MH475907.1), (MK813911.1). (Table 1)

Ten candidates for cryII gene sequences (Table 1) are aligned with multiple alignments using clustalW in the Bioedit program (v7.0.9 Tom Hall) to obtain conservative sequences as a prerequisite for good primary design. The results of the alignment of the cryII gene sequences are the conservative region in the CDS (coding DNA sequence) region. The CDS or ORF (open reading frame) sequence is the part of the gene that codes for amino acids in producing proteins. CDS regional base sequences were chosen because they have homologous sequences with other species but have unique bases (Furuno et al, 2003). The results of the alignment of the cry II gene sequences are made by

consensus sequences which are used as a template for primer design.

3.2 Primer Design and PCR in Silico

DNA primer designed using Primer3plus software (<https://primer3plus.com>.) was made by consensus sequences (Figure.1). Obtained ten potential primers candidates who can amplify the cryII gene. one primer was chosen that was deemed to meet the requirements of designing a good primer with a GC content of 55% and a Tm (temperature melting) value between 59 - 60 °C. The resulting product is ± 500 bp (basepair) with a primer forward (cry2F) 5'-ATCTGGTCTCATAGGGGCGA-3' sequence and a reverse sequence (cry2R) 5'-CGAGCTGTCGTGTTGCTTTG-3' (Figure 1).

Primers with Tm too high above 70 °C will easily experience mispriming at low temperatures. Primary pairs should not have a high Tm temperature difference between forward and reverse (Borah, 2011). As for the percentage of GC from the ten primary ranges between 50-60%. GC content plays a role in increasing primary stability. Strong hydrogen bonds in base pairs G and C cause primers to be more stable to stick to the template, so GC content is recommended to a range of 40% to 60% (Lin et al. 2005).

Annotation of cryII gene sequences using SnapGene software showed that CDS sequences

began with an initial codon with ATG code with a nucleotide length of 1910 base pairs with a total of 633 amino acids. In the amino acid sequence, the start of the codon began with the amino acid methionine (UAG). This can provide complete gene information in a genome. This indication must be proven by expressing the gene into *Escherichia coli* BL21 as a strain commonly used at gene expression levels (Seprianto and Febriana, 2018). Primer selection cry2F/cry2R was strengthened by PCR analysis in silico using SnapGene software with amplification regions that can be attached to the cryII gene CDS sequence template (Figure 2).

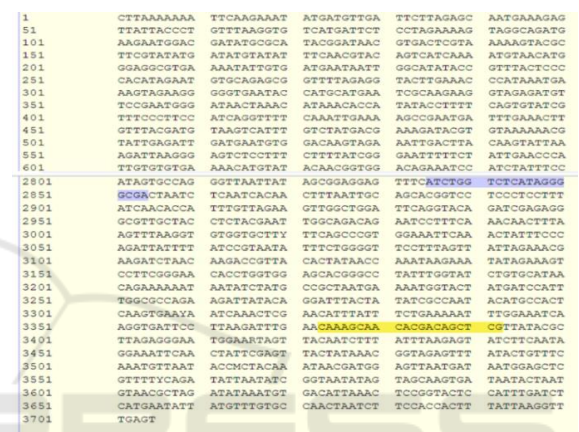


Figure 1: Consensus sequences of cryII gene used as a template for primer design.

Table 1: Ten candidates for cryII gene sequences from GenBank.

No	Description	Max score	Total score	Quary cover	E-val	Per Ident	Accession
1	<i>Bacillus thuringiensis</i> insecticidal crystal protein (cryII) gene. complete cds	3509	3509	100%	0.0	100 %	AF047038.1
2.	<i>Bacillus thuringiensis</i> strain SY49.1 cry2Aa gene. complete cds	3470	3470	99%	0.0	99.97%	KX243304.1
3.	<i>Bacillus thuringiensis</i> strain ly30 cry2Aa gene. complete cds	3469	3469	99%	0.0	99.74%	AF433645.1
4	<i>Bacillus thuringiensis</i> strain T532 insecticidal crystal protein (cry2Aa) gene. complete cds	3465	3465	99%	0.0	99.74%	MH475905.1
5	<i>Bacillus thuringiensis</i> strain T414 insecticidal crystal protein (cry2Aa) gene. complete cds	3465	3465	99%	0.0	99.74%	MG983754.1
6	<i>Bacillus thuringiensis</i> strain T405 insecticidal crystal protein (cry2Aa) gene. complete cds	3465	3465	99%	0.0	99.74%	MG983753.1
7	<i>Bacillus thuringiensis</i> serovar tolworthi Cry2Aa (cry2Aa) gene. complete cds.	3485	3485	98%	0.0	99.74%	DQ064596.1
8	<i>Bacillus thuringiensis</i> cry2Aa (cry2Aa) gene, complete cds	3506	3506	98%	0.0	99.74%	AF273218.1
9	<i>Bacillus thuringiensis</i> strain T543 insecticidal crystal protein Cry2A gene, complete cds	3480	3480	98%	0.0	99.68%	MH475907.1
10	<i>Bacillus thuringiensis</i> strain T380 Cry2A protein (cry2Aa) gene, complete cds	3480	3480	98%	0.0	99.68%	MK813911.1

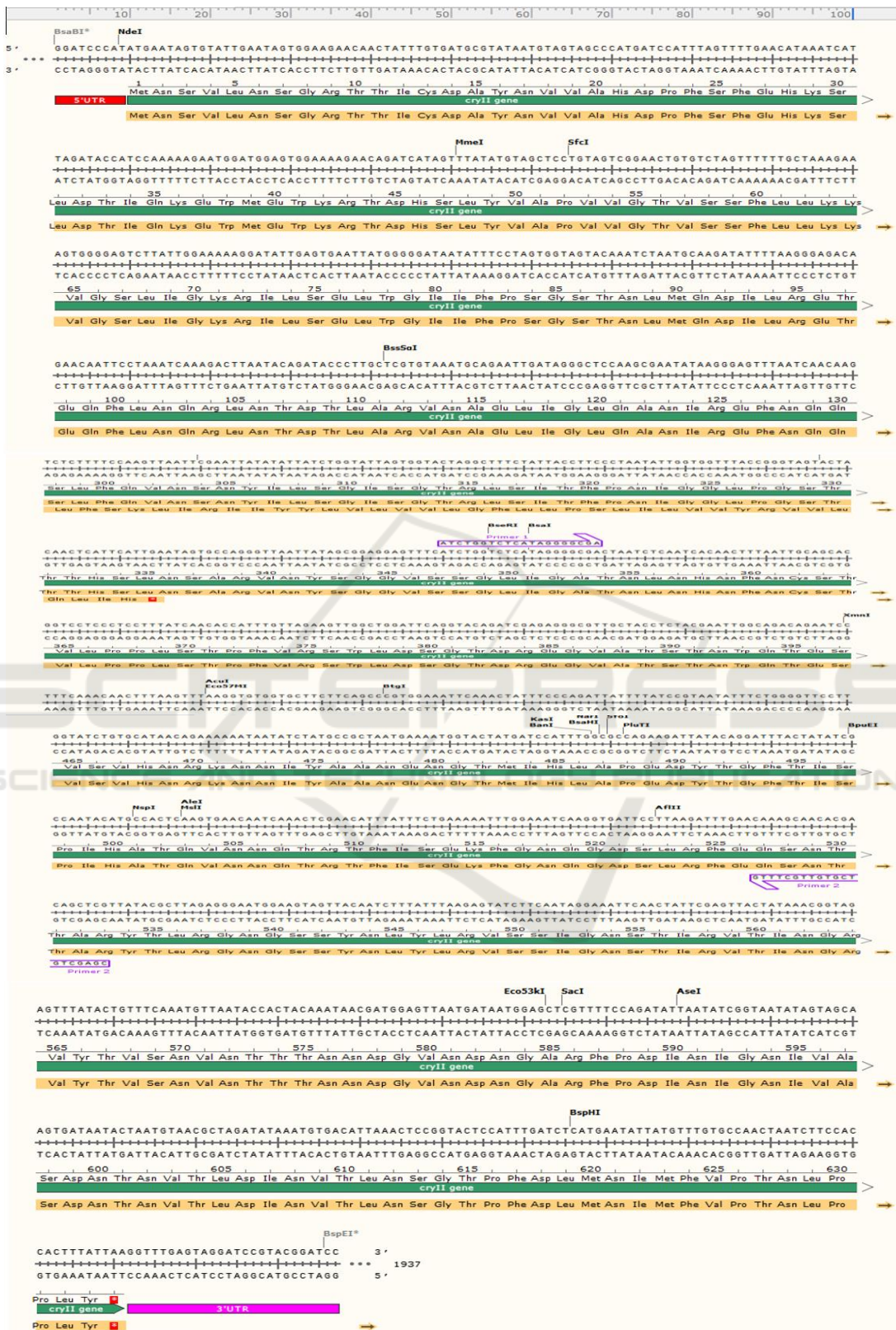


Figure 2: In silico PCR within an annotation of cryII gene in partial genome *Bacillus thuringiensis* (AF047038.1).

3.3 *Bacillus thuringiensis* Local Isolates

The bacteria used in this study were *Bacillus thuringiensis*. The bacterial isolate was originated from the InaCC (Indonesian Culture Collection) collection of the Indonesian Institute of Sciences (LIPI) with B327 and B432 local isolates (Figure 3). *Bacillus thuringiensis* was rejuvenated by growing on LB (Luria Bertani) medium and incubated for 24 hours. The choice of LB medium because it can stimulate the growth of spores and crystalline proteins from *Bacillus thuringiensis* (Mafazah and Zulaika, 2017). Isolation of the genome of *Bacillus thuringiensis* isolates B432 and B327 using Presto mini gDNA Bacteria Kit (Geneaid). Measurement of the purity and concentration of each DNA were performed using Nanoquant (TECAN Multimode Reader) (Table 2).

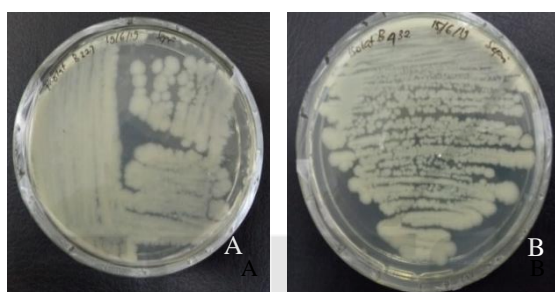


Figure 3: Colonies of *Bacillus thuringiensis* (A) B327 and (B) B432 isolates.

Table 2: DNA concentration and purity of genome Isolate B327 and B432.

Isolate	Absorb λ 260	Absorb λ 280	Concent (ng/ μ l)	Purity (λ 260/ λ 28)
B327	0,0128	0,0070	12,7	1,81
B432	0,0028	0,0015	2,80	1,88

The purpose of this measurement is to compare DNA extracts in units of nanograms per one microliter at a wavelength of λ 260/ λ 280 (ng/ μ L) based on absorbance values. Absorbance values are useful for detecting contaminants such as proteins, salts and polysaccharides that can inhibit DNA amplification (Kurniawati and Hartati, 2018). The ratio at a wavelength reading of λ 260/ λ 280 nm of 1.8 indicated that the extracted DNA has high purity in the absence of protein and phenols (Latif and Osman, 2017). The purity of DNA Bt strain B327 and B432 local isolates using nanoquant with a mark of 1,88 and 1,99 (Table 2). The λ 260/ λ 280 ratio with a range of 1.8 to 2.0 does not indicate significant contamination (Pervaiz et al., 2011).

3.4 Detection of CryII Gene by PCR Gradient

Optimization of annealing temperature (T_a) for detection cryII genes from *Bacillus thuringiensis* strain B327 and B432 local isolates by PCR gradient. A Gene amplification uses cry2F/cry2R specific primers with temperature melting (T_m) values between 59-60°C. The best annealing temperature is usually 2 – 5 °C below T_m . T_m is the temperature at which half of the DNA molecule is denatured. At too high a temperature causes specific primer attachment but the concentration of the amplicon obtained is very small so that the annealing temperature is very critical in the target DNA amplification process. While the temperature is too low causing the target DNA band expected results are not specific. A very high annealing temperature will result in inadequate DNA-mold primer hybridization resulting in low PCR products, while too low T_a will lead to unspecified amplification caused by the possibility of a primary attachment error on the DNA template. (Santoso et al., 2015).

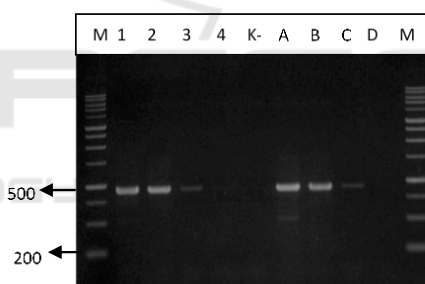


Figure 4: The result of Annealing temperature optimization for cryII gene amplification. (M). DNA Marker 100 bp Ladder. (sample 1-4) DNA isolate B432 (1) Ta 50°C, (2) Ta 54°C, (3) Ta 56°C, (4) Ta 60°C. (sample A-D) isolate B327, (A) Ta 50°C, (B) Ta 54°C, (C) Ta 56°C, (D) Ta 60°C. (K-) negative control.

The DNA band were successfully amplified with a size of \pm 500 bp with annealing temperature (T_a 50 °C and 54 °C), but at annealing temperature (T_a 56 °C) produced a thin DNA band. At annealing temperature of 60 °C, no ribbon was generated (Figure 4). These results indicate that the primer used is specific. The more specific the primer used, the fewer DNA fragments produced at the time of target DNA amplification. It was assumed that the high annealing temperature resulted in the low productivity of PCR. The temperature required for attachment of this primer depends on the base composition, length, and DNA concentration.

Primers candidate optimization involves optimization in annealing temperature (Ta) using PCR gradients and optimization of primary concentrations. In addition to primers, optimization of the PCR reaction was also carried out to check the minimum detection and quantification of nucleic acids in the reaction, and this requires work in the laboratory to produce a good PCR test (Saraswati et al., 2019).

4 CONCLUSIONS

This study used local isolate of *Bacillus thuringiensis* (B432 and B327) obtained from the InaCC (Indonesian Culture Collection). The genomic DNA purity of isolates B432 and B327 with A260/280 ratios are 1.88 and 1.81. Detection of CryII gene was analyzed using the Polymerase Chain Reaction (PCR) method, with the most optimum annealing temperatures of 50 °C and 54 °C. Using primers (cry2F) 5'-ATCTGGTCTCATAGGGGCGA-3' reverse sequences (cry2R) 5-'CGAGCTGTCGTGTTGCTTTG-3', amplification was successful for partial cryII genes with a DNA band size of ± 500 bp.

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