Primer concentration and Pre-denaturation Time Effect on *cyt-K Bacillus cereus* Detection using Real-Time PCR Method

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Abstract. Foodborne disease is a global threat that can affect all sections of society, both in developed or developing countries. *Bacillus cereus* is a Gram-positive bacteria that can cause food poisoning disease in humans. [2] Real-Time PCR detection method is one of the molecular marker methods that has been widely recognized as a fast, reliable, sensitive and specific detection tool for detecting pathogenic bacteria. In previous studies, the optimum condition and formulas applied for *cyt-K* 2 primer pairs have been obtained using Real-Time PCR. The purpose of this study is to find out the best conditions work of the primer pair *cyt-K Bacillus cereus* on detecting bacteria target using variations of pre-denaturation time and primer concentration with Real-Time PCR method. The annealing temperature used for PCR is at 60°C with sample concentration 50 ng/µL of *B. cereus*. Real-time PCR detection of variations in pre-denaturation time and primer concentration obtained the best conditions for primer pair *cyt-K* work at minute 4 with a primer concentration of 10 pmol and successfully amplifying the target by producing a Ct value of *B. cereus* at 13.04. Based on the results of the study, the primer pair *cyt-K* were reproducible in detecting the target gene and in the further step, this research can be continued to developed a prototype detection kit for foodborne pathogen bacteria using Real-Time PCR method.

1 INTRODUCTION

Foodborne disease is one of a global threat that can affect all sections of society, both in developed or developing countries. [1] Poor food handling and processing can be a way for contaminants to enter the body, such as pathogenic bacteria. [2] Bacillus cereus is a Gram-positive pathogenic bacteria that can cause food poisoning disease in humans. This bacteria belong to the bacterial domain, class in bacilli and its presence can be discovered in various types of environments, such as in soil, water sources, and plants. [3] Formed in micro size makes bacteria presence invisible and easier to contaminated water and food by transmitting from one place to another. *Bacillus cereus* has the capability to sporulated in food and germinate in small intestine producing diarrhoeal toxins when enter the human body. Diarrhea and emesis are two types of food poisoning caused of *Bacillus cereus*. The difference occurs by different toxins produced in host body. [4] Several cases in Indonesia related to outbreaks so called (Extraordinary Events) caused of *Bacillus cereus* often contaminate school snacks and infect students. Thus, a fast detection method is needed to detect pathogenic bacteria contaminant in food. [5]

Real Time-PCR is a molecular method that is quite sensitive in detecting disease infections, including foodborne diseases. As a highly specific and sensitive instrument, RT-PCR has simplified and accelerated the detection process which is capable of detecting low concentrations and a small number of samples contamination possibility at an early stage. Real-Time PCR method enables each cycle of DNA amplification to be observed by detecting sequence of sample PCR product. Three main steps of PCR includes Initial Denaturation at the beginning and Denaturation, Annealing and Extension. Each of step play an important role in the PCR process to produce good results detection. Other than that, primer pairs that annealed to the template also contribute to make a better result of PCR product. [6]

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In previous studies, the optimum condition and formulas applied for *cyt-K* 2 *Bacillus cereus* primer pairs detection have been obtained using Real-Time PCR. Therefore, this study aims to determine the best conditions work of *cyt-K Bacillus cereus* primer pairs on detecting bacteria target using initial denaturation time and primer concentration variations with Real-Time PCR. In further step, this research can proceed to developed prototype kit detection for food using Real-Time PCR.

2 MATERIAL AND METHODS

2.1 Bacterial Culture

Samples of *Bacillus cereus* bacteria ATCC 10876 were cultured onto BHI (*Brain-Heart Infusion)* Agar with an ose sterile needle using streak plate method. Then, the agar was incubated at 37°C for overnight culture (24 hours). After the incubation, white and round colonies of *B. cereus* are formed. After that, single colonies were taken using an ose sterile and inoculated into 10 mL of BHI Broth medium. Afterwards, the incubation was carried out at 37°C under an aeration shaker 150 rpm for 16-18 hours. The results show a cloudy color change in liquid medium that indicates the growth of bacteria.

2.2 Gram-Positive Bacteria DNA Extraction

DNA extraction process were carried out using a commercial kit by Thermo Scientific GeneJET Genomic DNA Purification Kit based on protocol handbook for Gram-positive bacteria isolation. The kit consists of three types of buffers (Wash Buffer I, Wash Buffer II, Elution Buffer), Proteinase K, RNAse, lysis solution, collection tube and mini white column.

Cultured samples of BHI broth from previous step were pipetted 3 mL for isolation process. Isolation stage for Gram-Positive bacteria required an additional lysozyme solution contained Tris HCl, EDTA, and Triton X-100. The result of DNA sample extraction was kept in 1.5 mL eppendorf tube sterile and stored at -20°C. Afterwards, purity of DNA sample extraction were tested using the Nanovue Plus™ UV-Vis Spectrophotometer (GE Healthcare Life sciences) and also characterized using 0.7% agarose gel visualized under UV transilluminator.

2.3 *Cyt-K* Primer Pairs PCR Amplification

In this process, the annealing temperature of 60°C is used in accordance with optimum temperature that have been obtained from previous studies. Primer pairs annealing temperature was confirmed to define the optimum temperature for primer attachment during the annealing stage. This process was carried out using PCR amplification process using Applied Biosystems™ Veriti™ Thermal Cycle PCR and require in total 25 µL volume cocktail PCR containing 1 µL each forward and reverse primer gene cyt-K 2, NZYTaq II 2X Colorless Master Mix, 50 ng DNA template of *Bacillus cereus* sample from isolation stage, and (Nuclease Free Water) NFW. The programmed PCR ended with a final extension at 72°C for 7 minutes and the cycle was repeated for 35 cycles. Afterwards, PCR results were characterized through a 2% agarose gel electrophoresis process and visualized under UV transilluminator light. Measurement of the result is compared with a marker ladder to determine the length of the amplicon.

2.4 Primer Concentration and Initial Denaturation Time Variations

In this process, Real-time PCR assay was carried out by varying pre-denaturation time and primer concentration used to find the optimum condition works for primer pairs of *cyt-K* 2 in amplifying the target. Pure DNA culture of Bacillus cereus were used as positive controls. There are two types of negative controls, which No Template Control (NTC) containing all Real Time PCR reagents without DNA templates and a mixture of NFW with Master mix. Samples were amplified using 7500 *fast Real-time* PCR *system* (Applied Biosystem). The RT-PCR steps were repeated for up to 40 cycles. Variations used for pre-denaturation time were divided into three parts, 3, 4, and 5 minutes with primer concentrations of 5, 10, and 15 pmol in each assay.

3 Result and Discussion

3.1 Growth Cultures of *Bacillus cereus*

Bacillus cereus culture using growth media in this study aims to obtain pure cultures of sample as a template for PCR. *Bacillus cereus* was cultured using BHI Agar and Broth. On BHI Agar, *B. cereus* produced a large, round-shaped colonies and formed a white spores around them, as shown in **Fig. 1**. The agar was incubated for 24 hours (overnight culture) at 37°C. The temperature range between 30°-37°C is the optimum temperature for bacteria to grow. *B. cereus* bacteria can grow well in a temperature range of 10° - 42°C. BHI is a non-selective medium that is commonly used as a culture medium for various other organisms such as fungi, yeast, and bacteria, including *B. cereus* because it is able to provide optimal growth.

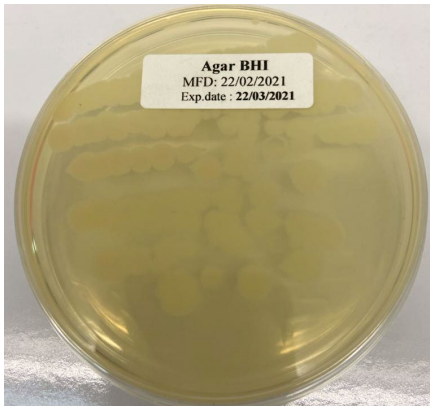


Fig. 1. *Bacillus cereus* colony in BHI Agar

Peptone and beef brain-liver infusion composition on agar, play a role in providing a source of carbon, nitrogen, vitamins, amino acids and elements needed for other bacterial growth factors**.** [7]

3.2 *Bacillus cereus* Isolation and Characterization

Real-time PCR assay requires a DNA template as a target to be amplified and produce PCR products. In order to obtain a template, isolation process from previous bacteria culture step was needed. DNA extraction in this study aimed to separate DNA from other contaminating materials such as proteins, carbohydrates, fats, RNA and other materials that can interfere result of PCR assay. The main principle in DNA extraction is lysis and extraction of tissue of bacterial wall and its components which then purified to to obtain a high quality and purity of DNA sample. [8] The results of DNA isolates were tested quantitatively using the *GE Nanovue UV-Vis Spectrophotometer* shown in the **Table. 1** below.

Table 1. Result of Bacillus cereus DNA Extraction

Sample	Concentration (ng/µL)	Purity (A260/280)
Bacillus cereus	78	1.866

The purity value of DNA isolate can be said to be pure when it is in the range of 1.8-2.05. If the result of purity value is less than 1.8, this indicates that sample still contains protein contaminants and if it is more than 2.0 contains RNA. [9] Based on the **Table. 1**, purity value of DNA isolates result is in a good purity range and free from protein and RNA contamination, which later can be used as a template for PCR method. Isolation results were then characterized using gel electrophoresis on 2% agarose as shown in **Fig. 2.** to confirm that there is DNA present in it and if it matched the size of whole genome size of bacteria. Electrophoresis process for DNA isolates is using a 1 kB size ladder.

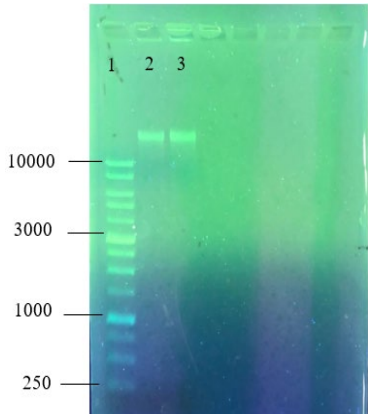


Fig. 2. Agarose electrophoresis DNA *Bacillus cereus*
(1) DNA Ladder 1 Kb; (2) *B. cereus* DNA Isolates
(3) *B. cereus* DNA isolates

Based on **Fig. 2.** results electrophoresis of *B. cereus* showed that the DNA bacteria was not degraded and the appearance of a single bright band above the marker ladder signify there are DNA present at size above 1 Kb. The whole genome size of *Bacillus cereus* in NCBI data is known to be 5,411,809 bp (base pair). From above evidence, both quantitatively and qualitatively, the isolation process has been successfully carried out and the DNA isolate is suitable for use as a PCR template and can proceed to the next stage.

3.3 Annealing temperature of *cyt-K* primer pairs for PCR

In previous research, [10] the optimum annealing temperature have been obtain at 60°C for *cyt-K 2 Bacillus cereus* with an amplicon target of 114 bp. The amplification and characterization of PCR was carried out in this research to validate that primer pairs work still eligible at optimum annealing temperatures to anneal specifically and produce an amplicon size on target. After PCR stage has been carried out, characterization of *B. cereus* PCR results was done using electrophoresis of 2% agarose gel and then visualized by illuminated under UV light as can be seen in **Fig. 3.**

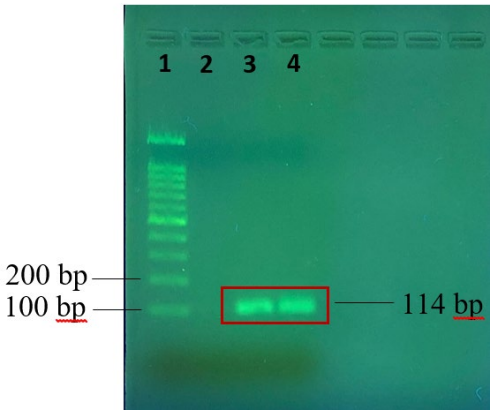


Fig 3. Characterization of *B. cereus* amplicon.
(1) DNA Ladder, (2) No Template Control (NTC),
(3) *B. cereus* Amplicon, (4) *B. cereus amplicon*

Based on **Fig. 3.**, it can be seen in well (3) and (4) that there is a single amplicon DNA band of *B. cereus* appeared with a size of 114 bp under UV light. This shows that the *cyt-K 2 B. cereus* successfully amplified during the PCR process using optimum annealing temperature, which was at 60°C. Primer pair also able to specifically produce an amplicon as target size 114 base pairs. In well (2), a No Template Control (NTC) as negative control shows that no band appears in agar. This can be means that the reaction is free from other contaminants that can be amplified. Thus, it can be concluded that the primers for the *cyt-K* 2 gene works and reproducible at the optimum annealing temperature 60 °C.

3.4 Real-Time PCR Variations of Initial Denaturation Time and Primer Contentration

Variations of Real Time-PCR assay was carried out in this study to obtain optimum conditions required for *cyt-K* designed primers can produce an optimal PCR products. Variations was done based on variabel conditions that significantly influence the success of the PCR process, such as variations in the length of initial denaturation time and primer concentration. Length of initial denaturation time used in this study were in 3, 4, and 5 minutes variations with primer pair concentrations vary between 5, 10 and 15 pmol. The concentration used for *B. cereus* template is 50 ng/µL for all conditions. The result of RT-PCR sigmoid curve results are shown in **Fig. 4-6**.

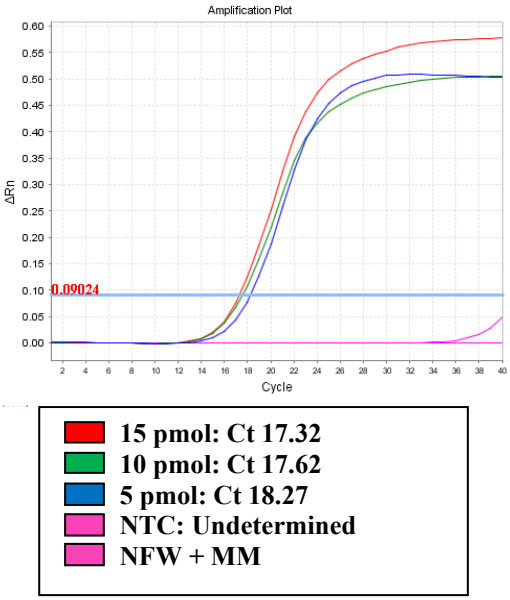
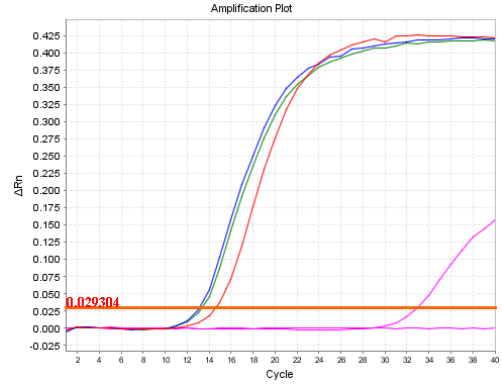


Fig. 4. Amplification Curve of *Bacillus cereus* Pre-denaturation time 3 minutes



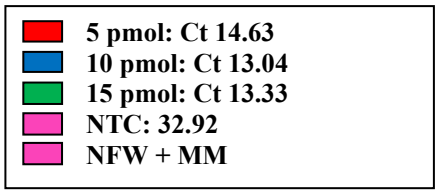


Fig. 5. Amplification Curve of *Bacillus cereus* Pre-denaturation time 4 minutes

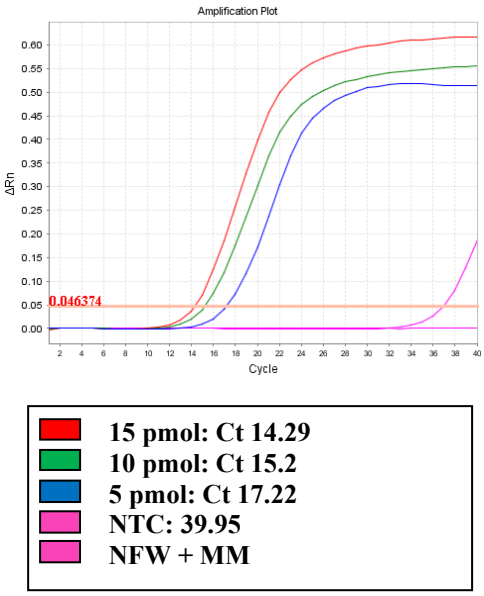


Fig. 6. Amplification Curve of *Bacillus cereus* Pre-denaturation time 5 minutes

Based on the amplification curve data above, the optimum condition obtained when 4 minutes of pre-denaturation time and 10 pmol primer concentration are used. This condition gave the smallest Ct value at 13.04, which indicates that cyt-K primer pair detect the target earlier than the other condition at cycle 13 with No template control (NTC) Ct value 32.92. Shifts in CT values can ideally occur in the dynamic range for real-time PCR is 7-8 fold for plasmid DNA, and a range of 3-4 for genomic DNA. Another negative control, contain NFW + Master mix PCR did not show any amplification signify that there is no other contaminants in the reaction. Ct value that occurred in NTC can be ignored if the value is more than 10 cycle compared to positive control. [11] Negative control is used as a control to determine the presence of possible contamination in the reaction.

The pre-denaturation or initial denaturation stage is a condition that initiates denaturation process in PCR to separate DNA double strands into single strands completely so that the primers can fully initiate extension. Imperfect denaturation process can produce a bad PCR products. Pre-denaturation time is affected by the percentage of GC content in the DNA template,

where more than 1-3 minutes is needed on templates that are rich in G-C content. [12]

On the other hand, primer concentration that is too low will cause the primer not stick to the target DNA, while a concentration that is too high will allow mispriming, the attachment of the primer to the DNA non-target area. [13] Thus, a pre-denaturation time of 4 minutes with a concentration of 10 pmol gave better results of cyt-K 2 gene *B. cereus* designed primer pair in detecting targets.

4 Conclusions

Primer pair *cyt-K 2 Bacillus cerus* succeed in amplifying target at optimum annealing temperature 60°C and produce an amplicon according target primer design from previous studies in 114 bp size. This can be concluded that primer pairs are reproducible to detect bacteria target. Variations of pre-denaturation time and primer concentration *cyt-K 2* primer pair using Real Time-PCR were obtained at 4 minutes with 10 pmol concentrations.

However, for further step research in developing a kit product detection, it is necessary to consider the cost efficiency and streamline the time consumed for production process. Based on Ct amplification result, a shift happened in a range between 13-18, which can indeed occured ideally, the dynamic range for real-time PCR is 7-8 fold for plasmid DNA, and a range of 3-4 for genomic DNA. Therefore, 3 minutes pre-denaturation time and primer concentration 10 pmol still also fine to used as a reference for building a prototype kit detection using Real-Time PCR method in near future.

Acknowledgements

This study was supported by Kemendikbudristek through the PDUPT scheme and contract 1/PG.02.00.PT/LPPM/V/2022. Our gratitude delivers to LPPM UNJ by the scheme Collaboration Industry under the contract agreement number 4/PK/LPPM/IV/2021. We also deliver our gratitude to the Indonesian Police Forces Canter-Forensic (Puslabfor Polri) whose facilitates the laboratory and all the equipment based on the MOU between UNJ and Puslabfor, We thank PT Kimia Farma as an industrial partner in the development of this detection kit. We would also like to thank the entire Salmonella team at the Center of Excellence for Pathogenic Bacteria Detection (PUI Pendeteksi Bakteri Patogen). Furthermore, we would like to express our gratitude to PT Sinergi Indomitra Pratama for providing the instruments for this study. We would like to acknowledge the support of IBD UTM to make this collaboration happen.

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