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Modification of CTAB incubation time in the isolation of DNA from *Staphylococcus aureus*

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Abstract
Background: DNA was an essential component in organisms that regulates biological activity. DNA analysis began with isolating pure, highly concentrated DNA, often used the CTAB method to isolate DNA from bacteria. DNA quality affected by incubation timed and temperature as well as its purity. The right incubation timed could optimize the degradation of proteins in the cell wall and maximize the released of DNA from the cell. DNA purity is good if the 260/280 absorbance ratio valued is 1.8-2.0. *Objectives*: This studied aimed to obtained the highest concentration and quality of DNA by modifying the incubation time of CTAB used *Staphylococcus aureus* DNA. *Materials and Methods*: This studied uses quantitative methods with the typed in experimental researched. The isolation stage included cell lysis with CTAB buffer and proteinase-K, purification stage with chloroform: isoamyl alcohol (24:1), DNA precipitation stage with isopropanol. *Results*: The average result of DNA purity obtained from the incubation treatment for 10 minutes was 1.89, for 20 minutes incubation was 1.46, and for 30 minutes incubation was 1.23. The average DNA concentration obtained from the incubation treatment for 10 minutes was 153 ng/μL, for 20 minutes incubation was 78.2 ng/μL, and for 30 minutes incubation was 26 ng/μL. Electrophoresis results at 10 minutes incubation time clearly visible DNA bands, 20 minutes incubation time DNA bands looked faint, and 30 minutes incubation time is not visible DNA bands. *Conclusions*: Based on the results of the studied, modification of CTAB incubation time with an incubation time of 10 minutes is the result of the highest concentration and quality of DNA using *Staphylococcus aureus* DNA.

Keywords
Cetyl trimethylammonium bromide, DNA isolation, Incubation time, *Staphylococcus aureus*.

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1. Introduction

Deoxyribonucleic acid (DNA) is a crucial component in all living organisms, governing all aspects of life by regulating biological activities within the body. In-depth understanding of DNA at the molecular level is the primary key to the advancement of scientific knowledge and technology. Molecular analysis processes focused on DNA begin with DNA extraction, aiming to produce DNA free

from contaminants and with high concentrations, thereby enabling more effective molecular analysis applications (Fatchiyah *et al.*, 2011).

Molecular analysis in microbiology involves crucial steps, including bacteriology that involves effective DNA isolation. Therefore, isolating DNA from both gram-positive and gram-negative bacteria is highly necessary. The *Cetyl Trimethylammonium Bromide* (CTAB) method is one of the most commonly used methods for isolating bacterial DNA. However, with the continuous advancement of technology, various products such as kits are available to aid the DNA isolation process (Fitriya *et al.*, 2015).

The DNA extraction process can be done through several methods, including conventional and modern methods using kits. Conventional DNA extraction can be done using the CTAB method (Mulyani *et al.*, 2011). The conventional DNA isolation method using CTAB is one of the methods that can be used to isolate DNA, although it requires more time. This method is usually used to isolate DNA from plant cells or fungi. Additionally, the CTAB method can be used easily and has a lower reagent cost (Heikrujam *et al.*, 2020). The CTAB method is recommended for DNA isolation due to its high purity level and the resulting higher DNA yield (Kumar *et al.*, 2012).

Factors influencing DNA quality include incubation time, incubation temperature, and DNA purity. If incubation time is too long, DNA can be damaged, but if it is too short, organelles will not break down. Optimal incubation time can maximize protein degradation on cell walls and maximize DNA release from cells. High incubation temperatures can cause DNA damage, while low temperatures will not destroy cell organelles. To obtain high-quality DNA samples, a combination of suitable incubation time and temperature is required. In addition to incubation time and temperature, DNA quality is also determined by its purity, which can be seen from the thickness of the gel electrophoresis strip and the absorbance ratio. To determine DNA purity, the absorbance ratio at 260/280 can be calculated using a spectrophotometer. This ratio should be within the range of 1.8-2.0 for pure DNA. If the ratio is lower than 1.8, the DNA is contaminated with proteins and polysaccharides, and if it is higher than 2.0, it is contaminated with RNA (Maftuchah *et al.*, 2014).

The research conducted by Koentjoro in 2021 on modified CTAB DNA isolation method using 10-minute and 30-minute incubation periods with human oral epithelial samples stated that longer incubation times can produce purer DNA. However, the research findings also discovered that the isolated DNA still contained contaminants such as proteins and phenol. The study by Retnaningati (2021) indicates that DNA extraction from melon leaf samples yields the highest concentration at

an incubation temperature of 65°C for 20 minutes, while the highest DNA purity is achieved at an incubation temperature of 70°C for 10 minutes.

This study aims to achieve the highest concentration and quality of DNA by modifying the CTAB incubation period using DNA from *Staphylococcus aureus*. The results obtained can enhance the efficiency of DNA isolation from bacterial samples using the CTAB method, which has been adjusted to include parameters such as DNA quantity (concentration) and quality (purity).

2. Materials and Methods

2.1. Types of research

This research is an experimental study conducted in February 2024 at the Molecular Biology Laboratory, Faculty of Health, Nahdlatul Ulama University, Surabaya, located at Jl. Raya Jemursari No. 51-57 Jemurwonosari, Wonocolo, Surabaya, East Java.

The samples used are pure cultures of *Staphylococcus aureus* (ATCC 25923) obtained from the Surabaya Health Laboratory (BBLK), located at Jl. Karangmenjangan No. 18, Surabaya. The sample size used in this study is the cultivation of *Staphylococcus aureus* bacteria in Luria Bertani (LB) liquid medium, followed by DNA isolation using the CTAB method. This test consists of three treatment groups with different incubation times at 65°C, namely 10 minutes, 20 minutes, and 30 minutes. The sampling was done using simple random sampling, meaning that the samples were taken randomly without considering specific categories within the population.

2.2. Research methods

The materials used include Nutrient Agar (NA), Luria Bertani (LB) media, TE buffer (Tris EDTA 50mM, pH 0.8), 10% SDS, proteinase-K, 5M NaCl, CTAB solution (composition: 2% CTAB (w/v); 0.1M Tris-HCl, pH 0.8; 0.5M EDTA, pH 8.0; 1.4M NaCl; 0.2% (v/v) β-Mercaptothanol), chloroform:isoamyl alcohol (24:1), phenol:chloroform:isoamyl alcohol (24:24:1), isopropanol, 70% ethanol, 1x Tris Acetate EDTA (TAE) buffer, 1% agarose, *Ethidium Bromide* (EtBr), loading dye, gel electrophoresis equipment (*Mupid-eXu*), UV-transilluminator (*PacificImage Electronics*), and a Nanodrop spectrophotometer (*Thermo scientific*).

This research began maintaining bacteria purity *Staphylococcus aureus* through Gram staining, coagulase testing, and Mannitol Salt Agar (MSA) testing to ensure the sample was pure *Staphylococcus aureus*. The next step was to grow the bacteria in NA slant media, incubated at 37°C

for 18-24 hours. Then, one bacterial colony was taken and placed in liquid LB media, incubated for 48 hours at 37°C until the solution became saturated or turbid.

The DNA isolation in this study began by adding 1.5 mL of bacterial culture to an Eppendorf tube. The culture was then centrifuged at 4,000 rpm for 5 minutes, after which the supernatant was discarded and the pellet obtained. The pellet was given an addition of TE buffer, 10% SDS, and proteinase-K, and then homogenized using a vortex for 30 seconds. The pellet was then incubated for 30 minutes at 37°C. Following incubation, the pellet was given an addition of 5 M NaCl and CTAB buffer, and then incubated again with varying incubation times of 10; 20; and 30 minutes at 65°C. The pellet was then given an addition of a chloroform: isoamyl-alcohol solution (24:1) of the same volume as the sample solution and homogenized by shaking the tube 10 times. The tube was then centrifuged at 8.000 rpm for 10 minutes. The topmost supernatant layer was transferred to a new Eppendorf tube and given an addition of a phenol: chloroform: isoamyl-alcohol solution (24:24:1) of the same volume as the solution. The suspension was homogenized by shaking the tube 10 times and centrifuged at 8.000 rpm for 10 minutes. The topmost supernatant layer was taken and transferred to a new Eppendorf tube, then given an addition of isopropanol of the same volume as the solution. The tube was centrifuged at 8.000 rpm for 10 minutes, after which the supernatant was discarded and the pellet washed with 500 μL of cold 70% ethanol. The tube was centrifuged at 8.000 rpm for 10 minutes, and then the supernatant was discarded and the obtained pellet dried until no alcohol remained. The obtained DNA was then rehydrated by adding 50 μL of TE buffer and stored at -20°C (Koentjoro *et al.,* 2021).

The bacterial DNA obtained through isolation is tested for its quality and quantity using a nanodrop spectrophotometer. The quantity of DNA is calculated by observing the absorbance value at a wavelength of 260 nm, where higher absorbance indicates the presence of visible fragments. Meanwhile, the quality of DNA is measured by comparing the absorbance values at wavelengths of 260 nm and 280 nm (Samsudin, 2020). Subsequently, the bacterial DNA samples obtained from isolation are semi-qualitatively tested using 1% agarose gel electrophoresis. The samples are placed in an electrophoresis chamber and added to 1x TAE buffer. Then, 10 μL of DNA sample and 2 μL of loading dye are added to each well, and electrophoresis is performed at a constant current of 100 Volts for 30 minutes. The resulting data is in the form of DNA purity and concentration of *Staphylococcus aureus*. The obtained results are analyzed descriptively quantitatively.

3. Results and Discussion

3.1. Result

The result of *Staphylococcus aureus* bacteria was conducted using Gram staining to ensure the sample used was pure of the bacteria. Figure 1 presents the result of the Gram staining observation at 100x magnification. Following this, a coagulase test was performed, yielding a positive result, which is presented in Figure 2. Finally, the MSA test yielded a positive result, which is presented in Figure 3.

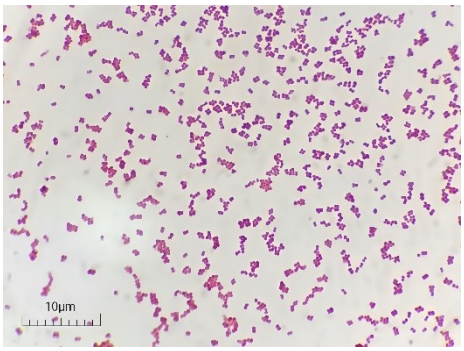

Figure 1. Gram Staining Result (1000x magnification)

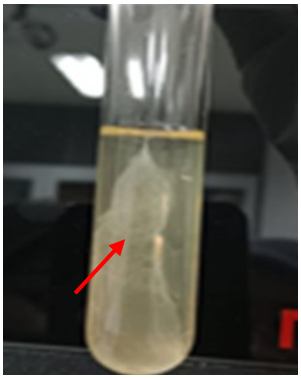

Figure 2. Positive Coagulase Test Result

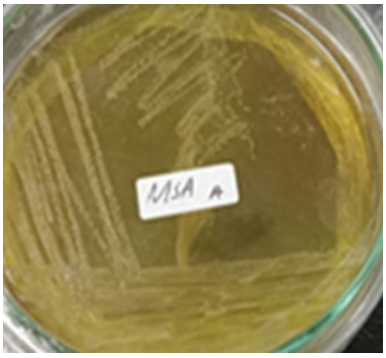

Figure 3. MSA Test Result.

The characteristic result of *Staphylococcus aureus* bacteria matches the research conducted by Dewi (2013), which shows that the Gram staining of *Staphylococcus aureus* samples reveals purple-colored bacteria with circular, grape-like clusters. In this study, the *Staphylococcus aureus* samples showed white-yellowish colony growth in MSA media surrounded by a yellow zone, indicating the ability to ferment mannitol. The coagulase test performed showed a positive result, with the presence of a clot at the bottom of the tube.

Bacterial cells obtained were isolated DNA using the CTAB method with different incubation times of 20; 30 and 30 minutes at 65 ℃. Each treatment was carried out 9 times. The effectiveness of the three variations in producing pure DNA is shown in Figure 4. The 10-minute incubation time produced an average 260/280 absorbance of 1.89 and a concentration of 153 ng/µL. Incubation time of 20 minutes produced an average absorbance of 260/280 of 1.46 and concentration of 78.2 ng/µL. Incubation time of 30 minutes produced an average absorbance of 260/280 of 1.23 and concentration of 26.3 ng/µL.

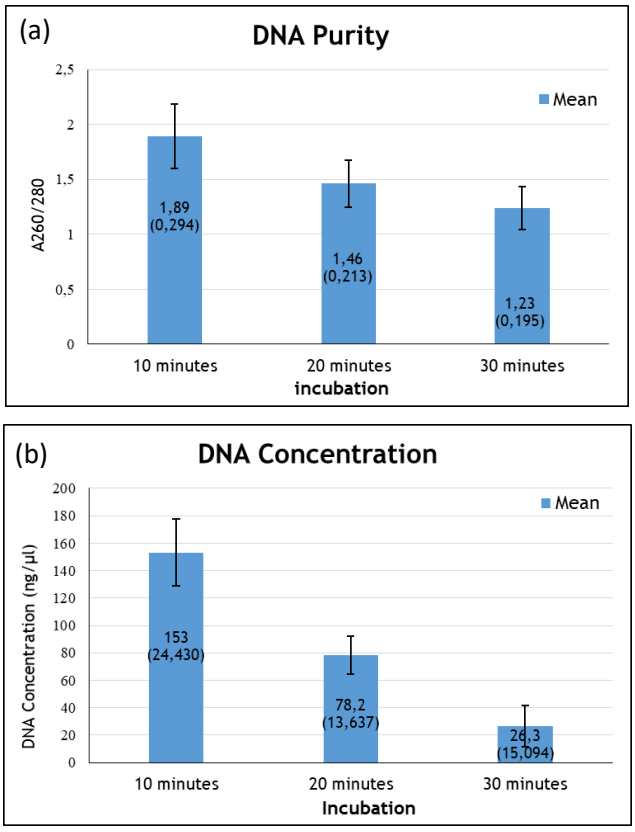

Figure 4. DNA quality and quantity test results (a) average DNA purity (b) average DNA concentration.

The DNA sample isolated from bacteria was tested for its quality using agarose gel electrophoresis. Figure 5 presents the entire DNA isolation result. At an incubation time of 10 minutes, 9 distinct DNA bands were observed. At an incubation time of 20 minutes, 6 DNA bands were observed faintly, whereas at an incubation time of 30 minutes, no DNA bands were observed.

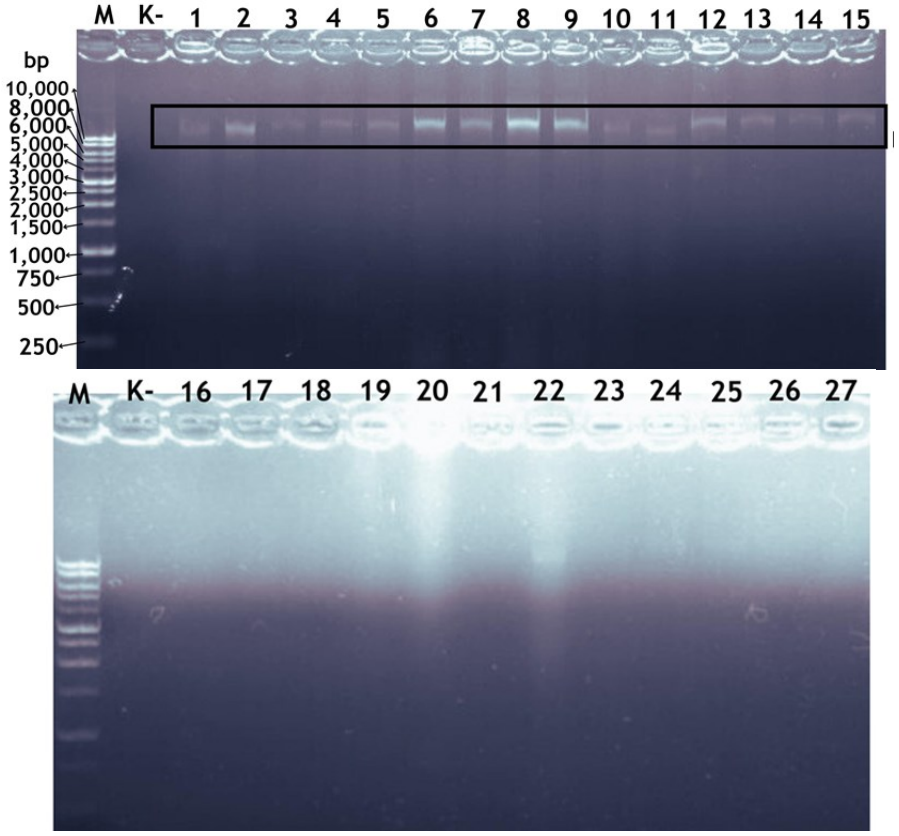

Figure 5. Electrophoresis results of DNA isolation from *Staphylococcus aureus* bacterial cells using CTAB with 10-minute incubation (rows 1, 2, 3, 4, 5, 6, 7, 8, 9), 20-minute incubation (rows 10, 11, 12, 13, 14, 15, 16, 17, 18), and 30-minute incubation (rows 19, 20, 21, 22, 23, 24, 25, 26, 27) on a 1% agarose gel; M: Marker (1kb); K-: Negative control (Nuclease-free water).

3.2. Discussion

The success of molecular analysis depends on the quantity and quality of the available samples. The amplification process that generates sufficient DNA for visualization through electrophoresis requires a sufficient amount of DNA in the sample. The quality and quantity of DNA can be measured using a nanodrop spectrophotometer. Additionally, the incubation time during the cell lysis process in DNA isolation is a crucial factor that can affect DNA damage. This incubation time plays a role in optimally degrading cell wall proteins and optimizing the release of DNA from cells (Maftuchah *et al.*, 2014). One conventional method still used in DNA isolation is CTAB. The CTAB method is used

for isolating DNA from plant leaves (Kumar *et al.*, 2012). In this study, samples of *Staphylococcus aureus* were used with varying incubation times.

The purity of DNA is measured using a spectrophotometer at an absorbance of 260/280 with a range of 1.8-2.0 (Maftuchah *et al.*, 2014). Quantification of DNA after a 10-minute incubation period shows an increase in DNA quality (purity) compared to 20-minute and 30-minute incubation periods (Table 1). The study results indicate that the average DNA purity after a 10-minute incubation period is 1.89, whereas after a 20-minute incubation period it is 1.46, and after a 30-minute incubation period it is 1.23. According to Yulia *et al.*, (2016), the low percentage of success in achieving purity is due to the presence of a DNA purity ratio still below 1.8. This indicates the presence of protein contamination in the DNA sample. DNA impurity can occur due to the presence of residual ethanol that is not fully removed during the drying process (Fatchiyah *et al.*, 2011). The average DNA concentration after a 10-minute incubation period is 153 ng/μL, after a 20-minute incubation period it is 78.2 ng/μL, and after a 30-minute incubation period it is 26.3 ng/μL. According to Pambudiono and Suarsini (2016), the difference in concentration is caused by the number of bacterial cells before the extraction process.

The incubation duration significantly affects DNA purity, as seen in the decrease in purity with longer incubation times. After a 10-minute incubation period, the average DNA purity is 1.89, but it drops to 1.46 after 20 minutes and 1.23 after 30 minutes. This decline in purity is primarily due to the increasing contamination of proteins and other substances in the DNA sample, which becomes more pronounced with longer incubation. DNA purity below 1.8 indicates protein contamination, which could be caused by residual ethanol not fully evaporating during the drying process. Similarly, the DNA concentration decreases with prolonged incubation time, with concentrations of 153 ng/μL; 78.2 ng/μL; and 26.3 ng/μL after 10; 20; and 30 minutes of incubation, respectively (Fang *et* al., 2020). The difference in concentration may be due to the degradation of DNA or inefficient extraction processes over extended incubation periods, which may not solely relate to the initial number of bacterial cells. Longer incubation may lead to the breakdown of DNA, thus lowering the overall concentration.

In line with the study by Naderlou et al. (2020), which demonstrated enhanced sensitivity and efficiency in detecting *Staphylococcus aureus* using modified magnetic nanoparticles through photometric systems, this research also indicates that the accuracy of DNA results is significantly influenced by the conditions and extraction methods employed. In Naderlou et al.'s research,

increased detection sensitivity was achieved thanks to the modifications made to the technology, which reduced contamination and improved result accuracy. Similarly, in DNA studies, contaminants such as proteins and solvent residues, like ethanol, can adversely affect the purity and concentration of DNA. Therefore, it is crucial to minimize contaminants and refine extraction methods to achieve more accurate results that align with purity standards, echoing the principles applied in modified photometric systems.

The DNA isolation result at a 10-minute incubation period in this study has a value ranging from 1.8 to 2. This result indicates that the isolated DNA is sufficiently pure (Koentjoro, 2021). Based on this study's findings, modifying the CTAB incubation time with a 10-minute incubation period results in the highest concentration and quality of DNA using *Staphylococcus aureus* DNA.

Based on previous research by Koentjoro (2021) on modifying the CTAB DNA isolation method on human oral epithelial samples, using 10-minute and 30-minute incubation times. The results showed that the best quality and quantity of DNA were obtained at the 30-minute incubation time. However, in this study, the best results were found with the treatment involving a 10-minute incubation time. This is due to the fact that bacteria belong to the prokaryotic cell group, which means they are low-level organisms from the *monera phylum*. Prokaryotic cells are a type of cell that lacks an internal membrane in the cytoplasm, both around the nucleus and within organelles, so the nucleus is directly in the cytoplasm. On the other hand, oral epithelial cells are categorized as eukaryotic cells. Eukaryotic cells have a nuclear membrane or nucleus that separates the contents within it. Typically, eukaryotic cells are possessed by multicellular living organisms. The structure of organelles in prokaryotes is significantly simpler compared to eukaryotic cells. Therefore, bacterial cell walls tend to be easier to break down (Hartono and Azimata, 2019).

The agarose gel electrophoresis result shows that after a 10-minute incubation, the DNA band is clearly visible, whereas after a 20-minute incubation, six faint DNA bands are observed. However, no DNA band is formed after a 30-minute incubation (Figure 4). Mulyani *et al.,* (2011) stated that DNA samples appearing as bright and thick bands indicate high concentration and purity. The bright and thick DNA bands observed after a 10-minute incubation also indicate high concentration and purity. The average DNA concentration is low during incubations of 20 and 30 minutes, resulting in faint or undetectable DNA bands. Additionally, contamination with proteins, RNA, or other substances can hinder DNA migration in the gel and make it difficult to detect (Mulyani *et al.,* 2011).

4. Conclusions

Based on the research results, it can be concluded that modifying the CTAB incubation time with a 10-minute incubation time yields the highest concentration and quality of DNA using Staphylococcus aureus DNA, with a DNA quality of 1.89±0.29 and a DNA concentration of 153±24.43.

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Author Contributions: Arisma Putri Awaliyah: Designing research, Performing laboratory wor, Analyzing data, Obtaining funding, Endah Prayekti: Research supervisor

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