

High-Integrity and High-Yield Genomic DNA from Staphylococcus aureus Using Cryogenic Cell Lysis

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Abstract

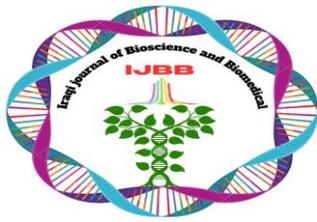
Molecular techniques are considered gold-standard tools for bacterial identification, detection of virulence genes, and whole genome sequence analysis. To achieve these goals, bacterial genomic DNA must first be extracted and purified. One of the main challenges in DNA extraction process is the bacterial cell wall, which is particularly tough and difficult to lyse, especially in Gram-positive bacteria.

Staphylococcus aureus is a Gram-positive pathogenic bacterium that causes a wide range of infections in humans and animals. It has a strong cell wall, which is hard to lyse. In this study, liquid nitrogen (-196°C) has been used for this purpose. The results showed high DNA concentration and purity by nanodrop spectroscopy; the concentration, from 3 ml bacterial culture, ranged between 800 and 2500 ng/μl with a purity between 1.8 and 2.1. In addition, DNA integrity was confirmed by gel electrophoresis. As well as the quality of purified DNA was confirmed by PCR. The *Staphylococcus aureus* nuc gene, which is widely used in molecular detection, was successfully amplified, and the obtained PCR product size was 279 bp. In the present research, the protocol used for *S. aureus* cell lysis by liquid nitrogen showed high efficacy, resulting in high DNA concentration and quality as well as reducing the time and cost.

Keywords: *Staphylococcus aureus*, Genomic DNA, Cell Wall, Liquid Nitrogen, Molecular identification

Introduction

Staphylococcus aureus is a round-shaped Gram-positive bacterium that normally lives on the human body as normal flora. However, it can behave as an opportunistic pathogen, causing a wide spectrum of infections ranging from minor skin ailments such as boils and impetigo to serious, life-threatening conditions, for instance, pneumonia, osteomyelitis, endocarditis, and septicemia¹⁻³. The potential of *S. aureus* to rapidly develop antibiotic resistance, particularly to widely used drugs such as methicillin, has become a serious concern in healthcare⁴⁻⁵.



Molecular techniques are considered golden tools that are currently widely used in microbiology laboratories for microbial identification and virulence genes detection. Conventional and real-time PCR are examples of the techniques which characterized by sensitivity and specificity⁶⁻⁷. Compared to traditional methods (for instance, culture, antigen detection, and microscopy), —often face several limitations. These include low sensitivity, time-consuming, slow growth, limited detection timeframes, and challenges in result interpretation⁸. Therefore, Molecular methods have been widely used in this regard.

In recent decades, there has been a great revolution in microbial genomic research due to the development of DNA sequencing technologies such as next-generation sequencing (NGS). These advancements have significantly reduced the cost and increased the speed of genome sequencing⁹⁻¹⁰. Nucleic acid extraction is the first step has to be done to obtain high-quality DNA or RNA for downstream molecular applications. Bacterial cell wall is the primary barrier that has to be lysed to release nucleic acids¹¹.

Staphylococcus aureus possesses a strong, thick cell wall measuring 20-40 nm, which displays typical characteristics of a Gram-positive bacterial cell wall that contributes to its resistance against environmental stresses and antimicrobial agents¹²⁻¹³. In addition, the bacteria made some modifications in their cell wall by adding an acetyl group to N- acetylmuramic acid (MurNAc) of the peptidoglycan. This modification make the bacterial cell wall resistant to lysis by enzymes such as lysozyme and lysostaphin¹⁴. Lysostaphin is more effective against *S. aureus* to break down bacterial cell walls, however, there are some isolates resistances to lysis by this enzyme and it is expensive¹⁵⁻¹⁶.

Treating living cells with liquid nitrogen (-196°C) led to rapid freezing of cells; consequently, ice crystals form inside cells lead to disruption of the cell wall and membrane. Therefore, liquid nitrogen can be used to lyse cells using a mortar and pestle to complete lysis and turn it into powder. This can overcome the problem of cell wall lysis resistance to lysis enzymes or incomplete cell wall digestion¹⁷⁻²².

This study aims to obtain high DNA yield and purity from *S. aureus* Gram-positive bacteria using liquid nitrogen and eliminating the need for enzymes such as lysozyme and lysostaphin, an enzyme that significantly increases the cost of DNA extraction.

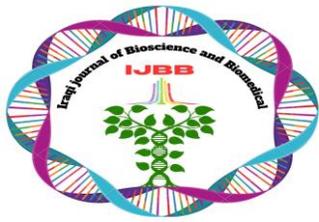
Materials and Methods

Microorganisms

The *S. aureus* isolates were provided by the College of Biotechnology, Al-Nahrain University, as work from previous students. Single colonies were isolated from each bacterial culture on Mannitol Salt Agar (MSA) and their identity were confirmed by VITEK 2 COMPACT (BioMérieux, USA).

Culture Conditions

The *S. aureus* isolates were cultivated in Brain Heart Broth (Himedia/ India) and incubated at 37°C overnight with shaking.



DNA extraction

Liquid nitrogen was purchased from Zamzam Oasis company (Baghdad/ Iraq) and the Wizard Genomic DNA Purification Kit (Promega/ USA). The bacterial cells (3 ml culture) were harvested by centrifuging at 15,000 x g for 2 minutes, the supernatant was discarded and the cell pellets were washed two times with 50mM EDTA (Promega). Then cell pellets were placed in a pre-chilled mortar and pestle and then covered with liquid nitrogen.

The frozen pellet was crushed with the pestle until it became a fine powder. Then, the cell-lysed powder was dissolved in Lysis Solution (600 µl), mixed gently and placed in a water bath at 80°C for 5 minutes. Then, 200 µl of Protein Precipitation Solution was added to the mixture and mixed well by vortexing. After that, samples were placed on ice for 5 minutes and centrifuged at 15,000 x g for 3 minutes. The supernatant was collected in a 1.5 ml tube and mixed well with 600 µl of isopropanol. The mixture was centrifuged at 15,000 rcf for 2 minutes. Then, the DNA pellets were washed with 600 µl of 70% ethanol. The samples were centrifuged for 2 minutes at 15,000 x g rcf. The ethanol was aspirated, and the tubes were left at room temperature for around 15 minutes. Finally, the pellet was resuspended in 100 µl of Rehydration Solution and stored at 4°C.

DNA Quality Assessment

The DNA concentration and purity were assessed using NanoDrop2000 spectrophotometer (Thermo Fisher Scientific/USA). The DNA integrity was evaluated by running it on 1% agarose gel electrophoresis.

Molecular detection of *Staphylococcus aureus* by PCR

Molecular identification of *Staphylococcus aureus* was done using the following primers: Forward Primer: GCGATTGATGGTGATACGGTT, Reverse Primer: AGCCAAGCCTTGACGAACTAAAGC that target the *nuc* gene. The PCR reaction was carried out in a 20 µl volume. The reaction components are shown in Table 1.

Table 1: PCR master mix preparation

Component	Volume (µl)	Final concentration
Nuclease-free water	7	-----
Gotaq® Green Master Mix (2X)	10	1X
Forward primer, 10µM	0.5	0.2 µM
Reverse primer, 10µM	0.5	0.2 µM
DNA-template	2µl	< 250 ng

GoTaq® G2 Green Master Mix kit (Promega, USA) was used in this study. The PCR reaction was performed following the program in Table 2.

Table 2: PCR program

Steps	Temperature (°C)	Time (second)	Cycles
Initial denaturation	95	120	-
Denaturation	95	30	35
Annealing	54	30	
Extension	72	30	
Final extension	72	300	-

Agarose gel electrophoresis

Genomic DNA samples and PCR products were analyzed on 1% agarose gel in 1X TBE buffer. The electrophoresis was run at 60 V for 60 minutes. A DNA ladder was used as a molecular size marker. Gels were stained with ethidium bromide (0.5 µg/ml) and DNA bands were visualized under UV illumination using a gel documentation system (Bio-RAD, USA).

Results and Discussion

Staphylococcus aureus isolates were cultured in Brain Heart Infusion (BHI) broth. Whereas the bacterial isolates cultivated on Mannitol Salt Agar (MSA), which is widely used as a selective and differential medium¹⁸⁻¹⁹. After incubation at 37°C overnight with shaking, the *S. aureus* colonies and the medium appeared yellow in color (Figure 1). Because *S. aureus* can ferment mannitol and generate acid as a result the color of the bacterial colonies and MSA medium turns to yellow²⁰.



Figure (1): *Staphylococcus aureus* culture on Mannitol Salt Agar incubated overnight at 37°C.

DNA extraction and quality assessment

The DNA extraction from *S. aureus* isolates using liquid nitrogen was highly efficient to get high concentration and purity without using lysis enzymes. The bacterial genomic material quantity and quality were determined by nanodrop spectroscopy. The concentration of DNA ranged between 800 to 2500 ng/ μ l (Figure 2) and the purity values were between 1.8 to 2.1. Since the RNase enzyme was not used in this study, the value of the ratio is close to 2.1. The A260/A280 absorbance ratio is commonly used to evaluate the purity of DNA and RNA samples²¹. The obtained DNA absorbance curves showed the typical pattern with a peak at 260 nm (Figure 3).

The reasons for obtaining high yield DNA using liquid nitrogen were due to complete lysis of the bacterial cell wall and membrane and release of all cell components, while lytic enzymes could not completely digest bacterial cells, leading to a reduced DNA concentration. Another advantage of using liquid nitrogen in bacterial cell lysis is that it does not require an incubation period and is also inexpensive compared to the method of using enzymes. No need for incubation time like lytic enzymes such as lysozyme, which need incubation time at a certain temperature²²⁻²³.

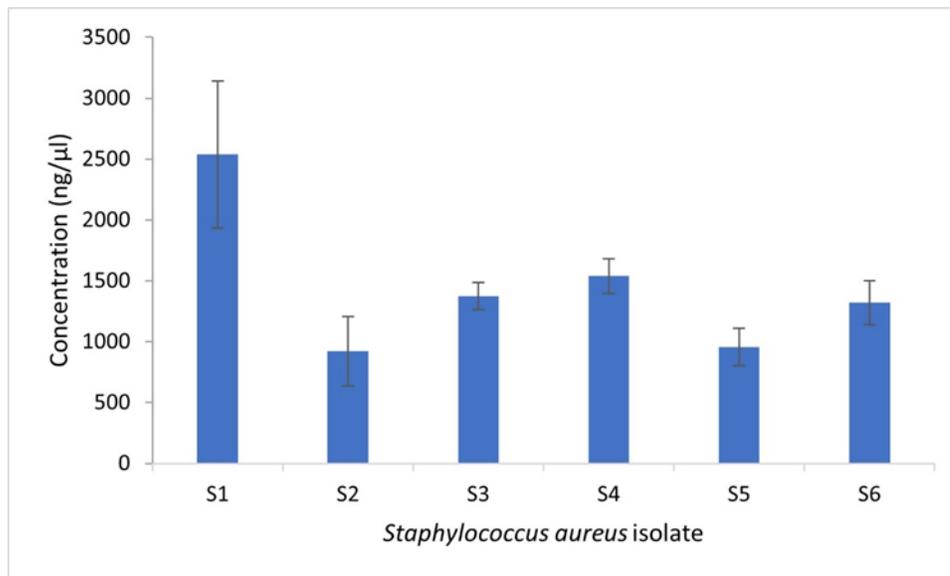


Figure (2): DNA concentration obtained from three *S. aureus* isolates whose cell walls were lysed by using liquid nitrogen.

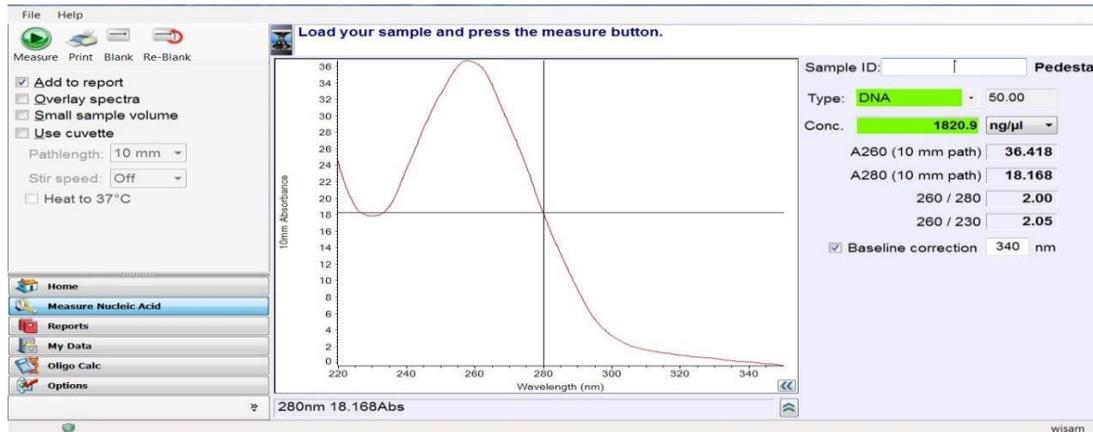


Figure (3): Assessment of extracted DNA by NanoDrop 2000/2000c Spectrophotometer shows a typical nucleic acid curve.

The extracted DNA integrity was tested on agarose gel electrophoresis. The result showed high-molecular-weight sharp bands without smear, indicating good DNA quality and there is no degradation (Figure 3). DNA with high purity and integrity is essential to perform PCR and DNA sequencing²⁴⁻²⁵.

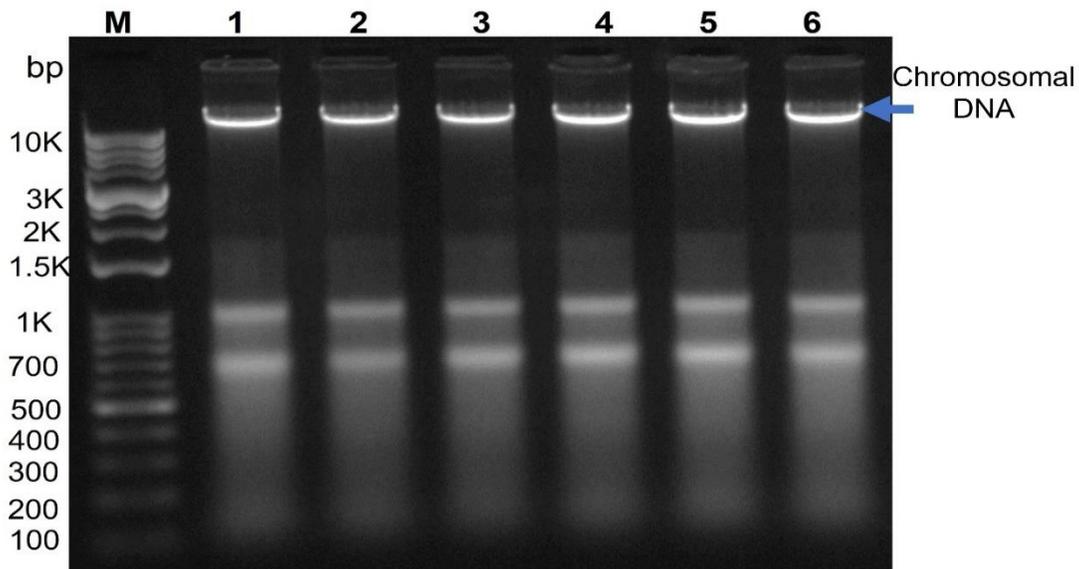


Figure (3): Agarose gel electrophoresis for *S. aureus* extracted genomic DNA. Lane M: DNA ladder and lanes 1-6 genomic DNA.

Molecular identification of *Staphylococcus aureus*

Staphylococcus aureus identity was confirmed by using specific primers to amplify the *nuc* gene by PCR. The PCR result (Figure 4) shows the right target size, which is 279 bp that confirm amplification of the target gene. PCR targeting the *nuc* gene was successfully used to confirm the identity of *S. aureus* isolates. The *nuc* gene is highly conserved among *S. aureus* strains, making it a robust molecular marker²⁶. Unlike conventional biochemical tests, which may yield inconclusive results due to phenotypic variations, PCR-based *nuc* gene detection offers high specificity, sensitivity, and rapid identification²⁶⁻²⁷.

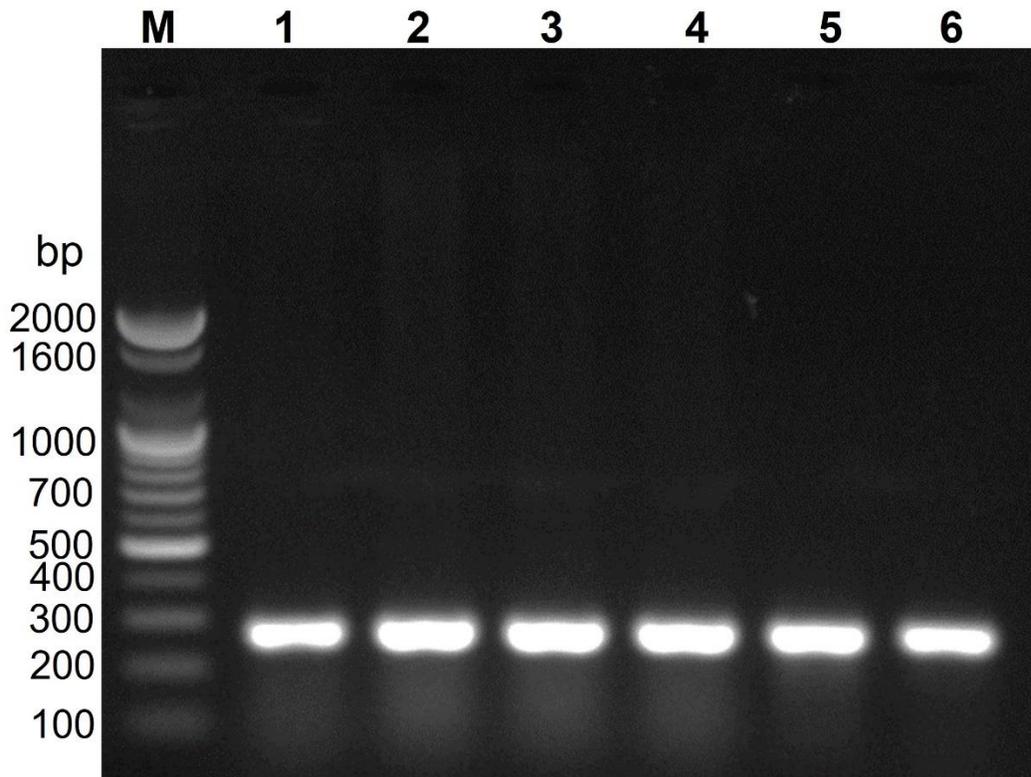
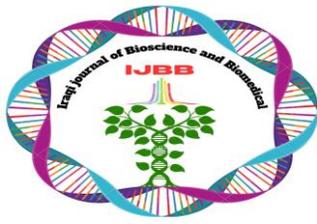


Figure (4): *S. aureus nuc* gene (279bp) amplified by PCR separated on 1% agarose gel. Lane M: DNA ladder and lanes 1-6 *S. aureus* isolates.

Conclusions

Staphylococcus aureus has a strong cell wall, which is considered a big challenge for cell lysis, even with using lytic enzymes, the DNA yield is very low. In the present research, we showed that using liquid nitrogen for lysing the cell wall of the bacteria was a very efficient way. Through which high DNA concentration was achieved combined with purity and integrity. This DNA was used successfully to amplify the *nuc* gene of *S. aureus* by PCR.



Acknowledgments

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Author's Declaration

- We hereby confirm that all the Figures and Tables in the manuscript are original and have been created by us.
- We have obtained ethical clearance for our study from the local ethical committee at [Al-Nahrain University/College of Biotechnology]. This approval underscores our commitment to ethical research practices and the well-being of our participants.
- Ethical Clearance: The project was approved by the local ethical committee at [Al-Nahrain University/College of Biotechnology], ensuring adherence to ethical standards and the protection of participants' rights and welfare.

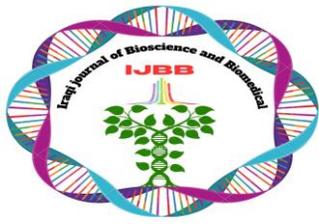
Author's Contribution Statement

[First Author]: Conducted experiments, collected literature review and wrote the draft of the manuscript.

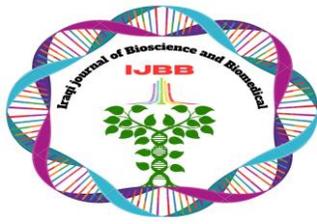
[Second Author]: Designed the study, supervised the research project, conducted some experiments and revised the manuscript.

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