



The impact of Glucose and Sodium Chloride on the Biofilm Formation of *Pseudomonas aeruginosa* & *Staphylococcus aureus*

Estabraq A. Mahdi

Abdulwahid B. Al-Shaibani

Nedhaal S. Zbar

Department of Medical and Biological, College of Biotechnology Al-Nahrain University, Baghdad, Iraq.

adelestabraq@yahoo.com

Department of Medical and Biological, College of Biotechnology Al-Nahrain University and Al-Farabi University College, Baghdad, Iraq

Department of Medical and Biological, College of Biotechnology Al-Nahrain University, Baghdad, Iraq.

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Abstract

The aim of this research is to evaluate the effect of glucose and sodium chloride on biofilm formation by bacteria causing wound infection. For this purpose, 1% and 2% concentration of each of glucose and sodium chloride were used to test the biofilm formation potential of *Staphylococcus aureus* and *Pseudomonas aeruginosa*, which were the most common abundant bacteria that cause infection by biofilm. Each of the concentrations was kept in contact with the pathogenic bacteria for 24 hours. After the period of incubation, the concentration of 1% of glucose enhanced moderate biofilm formation capacity for (66% and 80%) on both bacteria respectively. The concentration of 2% glucose, on the other hand, led to a weak biofilm for 33% and 20% on both bacteria isolates respectively. In respect to the effect of sodium chloride, no isolate was able to form neither moderate nor strong biofilms. Nonetheless, all isolates succeeded in forming weak biofilms at 2% sodium chloride, while treatment with a concentration of 1% sodium chloride led to inhibited biofilm formation for 43% of isolates. Besides, *Pseudomonas aeruginosa* isolates were able to form moderate biofilms in the presence of 1% concentration of glucose, and weak producers in the presence of 2% glucose concentration. The isolates succeeded in forming strong biofilms at both 1% and 2% sodium chloride.

Keywords: Biofilms, Wounds Infections, Bacteria.



1. Introduction

Bacterial biofilms are regarded as the major cause of chronic wound infections. Their high resistance against antibiotic treatments makes their annihilation more challenging. The majority of chronic wound infections are a result of biofilms that seriously slows down the process of wound healing [1]. Biofilm formation shows a protective style of growth that permits the microorganism to stay alive in various environments and capability of biofilm formation that is among the defense strategies of *Staphylococcus aureus*. Typical antibiotic treatments are generally impractical in eradicating bacteria that are embedded in biofilms, as the latter are naturally unsusceptible to the immune responses of a host [2]. Biofilm construction is a secure adjusting type of thriving that allows bacteria not only to survive in inhospitable conditions like those of a human body but also to propagate and spread their requirements, which are facilitated by their molecular crosstalk known as Quorum Sensing (QS) [3]. The main inconsistency of chronic infections is their inscrutability, which are in most instances associated with biofilm formation, as the National Institute of Health in the USA estimates that biofilms are linked to 80% of all bacterial infection. The mechanisms of biofilm resistance to antimicrobial compounds include physical or chemical diffusion barriers to antimicrobial penetration into the biofilm. For example, ciprofloxacin binds to certain components of *P. aeruginosa* biofilm, thus, the biofilm acts as a barrier to antimicrobial agents [4]. Dead remnants of neutrophils in addition to other immune cells found to act as a biological network that promotes the development of biofilms, and neutrophils within biofilms to produce various inflammatory intermediaries, which cause tissue injury. Biofilm development is organized by the expression of polysaccharide intracellular adhesion molecule that facilitates cell to cell adhesion. The matrix of bacterial biofilm is made of different polymers such as polysaccharide, proteins, and extracellular DNA (eDNA). The formation of biofilms is a procedure that goes through multiple stages, the first of which is a provisional attachment to the surface. Afterwards, the real attachment is induced by particular bacterial adhesions called microbial surface components, which recognize adhesive matrix molecules. Then bacteria agglutinate and form extracellular polymeric substances during the step of accumulation [5]. This process of biofilm construction relies on various factors such as the properties and the nature of the carbon source, its concentration, pH, ionic strength, and temperature [6]. Although researchers have attempted to refine the requirements needed for the construction of biofilms by *Staphylococcal* and *Pseudomonas* isolates, certain factors have yet to be sufficiently studied, including the ideal sugar and salt concentrations, and the richness of medium. Some researchers have studied the impact on the biofilm phenotype by using glucose supplementation along with Tryptone Soy Broth (TSB). While others have comprehensively elucidated sodium chloride (NaCl) dependence of biofilms in *S. aureus*, but their quantitative analysis and classification, according to the parameters of biofilm formation, are not clear and cannot be repeated in every research laboratory. Therefore, there is a desperate need for a simple and generally agreed-upon criterion for laboratory biofilm production by clinical isolates of *S. aureus*. As far as we are aware, investigators generally do not opt for a concentration of sugar and salt that is beyond 1% and do not conduct enough studies about how the features of *S. aureus* biofilms are affected by culture medium, fixation, elution, and the supplementation of various degrees of salt and sugar to a wider level of concentration [7]. On the aforementioned grounds, we aim in this study to establish a consensus method to attain the maximum possible in vitro biofilm construction using

clinical isolates from wound infection bacteria consuming the supplementations with the suitable concentration of glucose and sodium chloride.

2. Materials and Methods

2.1. Clinical Isolates and Penotypic Identification

Swabs samples were taken from ninety surgical wounds of patients attending Medical City Hospital in Baghdad, for the period from July to September 2018. The samples were taken by the attending physician of the hospital and collected using sterile sticks with cotton swabs in test tubes. Every specimen streaked on nutrient Agar, mannitol salt agar (MSA), blood agar and MacConky agar. Then, after 24 hours of incubation at 37 °C, the isolates were identified according to the microscopic characterization, colony morphological features, and gram staining. Various biochemical tests including the catalase, coagulase, oxidase and DNase test and others were carried out according to Bergey's Manual [8].

2.2. Detection of Biofilm in Presence Different Concentration of Glucose and Sodium Chloride

Biofilm formation was determined by microtiter plate method (MTP) [9], which is regarded as the standard and most frequently applied method in the investigation of biofilm synthesis disclosure. The microtiter plate method was applied to bacteria isolates by incubating overnight in nutrient broth at 37 °C. Bacterial isolate suspension that was equal to the McFarland turbidity standard no. 0.5 was inoculated in the nutrient broth separate sterile, polystyrene, flat-bottomed tissue culture static condition 96-well plate. The nutrient broth was supplemented with 1% and 2% concentrations of glucose and 1% and 2% concentrations of sodium chloride (NaCl). Subsequently, 200 µL of the inoculum was moved to the inspection wells of a sterile microtiter plate, which was related to an inoculum of approximately 5×10^6 cells per well. The lids covering each plate were supplied by the manufacturing company. Ultimately, the inoculated assay plates were incubated overnight at 37 °C, where the negative control wells consisted of the sterile nutrient broth alone, and the positive control wells had both the nutrient broth along with bacterial cells without glucose or sodium chloride. Each well was decanted and washed three times using Phosphate-Buffered Saline (PBS) to remove any non-adherent bacteria. The washed plates were first incubated for a minimum period of one hour at 60 °C to stain fix adherent cells. The purpose of this step was to lessen the variability caused by the biofilm loss during the staining procedure. After the fixation, 200 µL of 0.1 % Crystal Violet (CV) dissolved in distilled water was introduced to every well at least 15min. for staining. Afterwards, the reaction has been ended; Phosphate-Buffered Saline was used repeatedly 3 to 4 times to wash. Then every well was introduced with 200 µL of 95% ethanol for 10 minutes. The assays were all done in triplicates. The amount of crystal violet extracted by the ethanol in each well can be directly quantified spectrophotometrically by measuring the optical density (OD) at 630 using an appropriate ELISA reader [9]. Table (1) displays the classifications depending on the values of optical density (OD) that were obtained from various strains of *Staphylococcus* and *Pseudomonas* and were utilized for the sake of data simplification and calculation [10].

Table 1: Bacterial Adherence Classification according to the Method of Microtiter plate

Mean OD 630 nm	Biofilm Construction Adherence
$OD \leq OD_c$	Non-adherent
$OD_c < OD \leq 2OD_c$	Weakly adherent
$2OD_c < OD \leq 4OD_c$	Moderately adherent
$4 OD_c < OD$	Strongly adherent

3. Results and Discussion

3.1.1. *Staphylococcus* Isolation and Identification

Thirty isolates were isolated 90 samples of wound, which constitute 33% of the total number, were able to grow in mannitol salt agar, which is regarded as a selective and differential medium for the *Staphylococcus* species. The colonies looked round, smooth, raised, mucous and glistening, which indicates that these isolates belong to the genus *Staphylococcus* [11]. Certain isolates had the capability of fermenting mannitol, generating splendid production, and forming vast golden colonies surrounded by broad yellow fields, causing the medium color to alter from pink to yellow [8]. This golden color feature of *S. aureus* was accounted for by the carotenoid pigment (Staphyloxanthin) according to Clauditz *et al.* [12]. The gold pigment is an eponymous feature of the human pathogen *S. aureus* that shield the microbe from oxidation-based clearance [13]. After subjecting the thirty isolates to Gram staining, the microscopic examination showed that their cells appeared cocci mostly arranged in grape- like irregular clusters. The 30 isolates all gave a negative result to an oxidase test, which was carried out in order to distinguish genus *Staphylococcus* from genus *Micrococcus* that normally yields a positive response. In addition to that, the isolates all responded positively to the catalase test, which was performed to distinguish *Streptococcus* species that typically respond negatively to the test, from *Staphylococcus* species [14]. All mannitol fermenters were noticeably coagulase and DNase positive, and all *S. aureus* isolates provided positive responses to DNase and developed a beta hemolysis behavior on blood agar, as shown in **Table (2)**.

Table 2: Morphology and biochemical of the *Staphylococcus aureus* isolates

Test	<i>Staphylococcus aureus</i>
Gram stain	Gram-positive
Blood agar medium	β -hemolysis
Mannitol salt agar	Yellow colony
Catalase	Positive
Oxidase	Negative
Coagulase	Positive
Deoxyribonuclease (DNase agar)	Positive

3.1.2. *Pseudomonas* Isolation and Identification

A total number of 25 /90 isolates had the ability to grow on cetrimide agar plates and were suspected to belong to genus *Pseudomonas* cetrimide agar which differentiated *P. aeruginosa* from other species of *Pseudomonas* that could not grow on this medium. They were subject to further identification according to morphological characteristics and biochemical tests. Colonies of each isolate placed on nutrient agar showed different morphological characteristics of *Pseudomonas* species such as mucoidal growth, smooth shape with flat edges and elevated center, whitish or creamy color, fruity odor, and production of pyocyanin. On the other hand, colonies of *Pseudomonas aeruginosa* that grew on MacConky agar medium appeared pale in color and had irregular, oval, and large edges. On blood agar, *Pseudomonas aeruginosa* was able to perform hemolysis completely, which was a reasonable result in comparison with results demonstrated by Forbes *et al.* [15]. Microscopic analysis of *Pseudomonas* species revealed that the cells were bacilli; gram-negative; non-spore forming; and appeared single, in pairs, or short chains. These results were comparable to the reported morphological characteristics of *Pseudomonas aeruginosa* and agreed with Harley and Prescott [16], which certified the identification. Biochemical tests for *Pseudomonas* sp. were also made and their results indicated in **Table (3)** showed that these isolates gave a positive result for oxidase and catalase tests, which signified that they belonged o *Pseudomonas aeruginosa*.

Table 3: Morphological and Biochemical Characteristics of the *Pseudomonas aeruginosa* Isolates

Test	Result
Colony color	Green on Cetrimide agar
Cell shape	Bacilli
Gram stain	Negative
Catalase	Positive
Oxidase	Positive
Growth on King A	Positive
Growth on King B	Positive
Growth on cetrimide	Positive
Citrate utilization	Positive
Growth at 4°C in nutrient agar	Negative
Growth at 42°C in nutrient agar	Positive

The numbers and percentages of the microorganisms isolated from the patients 'wound infections were shown in **Table (4)**. These results were obtained according to the identification criteria mentioned for the bacterial isolates.

Table 4: Numbers and percentages of bacteria isolated from the wound infection patients

Types of isolates	Number of isolates	Percentages of isolates
<i>S. aureus</i>	30	33.3
<i>P. aeruginosa</i>	25	27.7
Other types	35	38.8
-	90	100

3.2. Effect of Glucose and Sodium Chloride on biofilm formation of the Isolates

This study showed a trend among the clinical isolates of *P. aeruginosa* and *Staph.aureus* to form a biofilm. All *S. aureus* isolates were able to form a weak biofilm in a nutrient broth without any supplementation, while in the presence of 1% glucose biofilm formation capacity was enhanced by 66% for *S. aureus* isolates, as shown in Table (5) and figure (1). It was noted in the results that glucose sugar, with a concentration of 1%, worked to increase the formation of the biofilm by 66%. While the concentration of 2% inhibited the formation of the biofilm by 16% and reduced the ability of isolates to the formation of the moderate biofilm by 33%. Whereas it increased the ability to form a weak biofilm to 50% at a concentration of 1%, but the increase did not significantly effectness. In respect to the effect of sodium chloride, it resulted by the inhibited the biofilm formation by 76% and 43% at a concentration of (1,2%). The results showed a decrease in the ability of the isolates formation of the weak biofilm to 23 and 56% for both concentrations respectively. Collectively, results revealed that 1% glucose was significantly better than other glucose concentrations and NaCl in improving biofilm formation by *S. aureus* isolates. Glucose has been shown to induce the multicellular aggregation step of biofilm formation [17]. Sugimoto *et al.* suggested that the treatment with a high concentration of NaCl enabled them to detach and yield *S. aureus* biofilm matrices [18].

Table 5: Effect of Glucose and NaCl on a Biofilm Development by *Staphylococcus aurous* after 24 Hours of Incubation

Number of <i>S. aureus</i> isolates (%)				
Media use	Non Biofilm	Weak biofilm	Moderate biofilm	Strong biofilm
NB without enhancer	0 (0%)	30(100%)	0 (0%)	0 (0%)
NB + 1% glucose	0 (0%)	10(33%)	20 (66%)	0 (0%)
NB + 2% glucose	5(16%)	15(50%)	10(33%)	0 (0%)
NB + 1% NaCl	23(76%)	7(23%)	0 (0%)	0 (0%)
NB + 2% NaCl	13(43%)	17(56%)	0 (0%)	0 (0%)

NB; nutrient broth

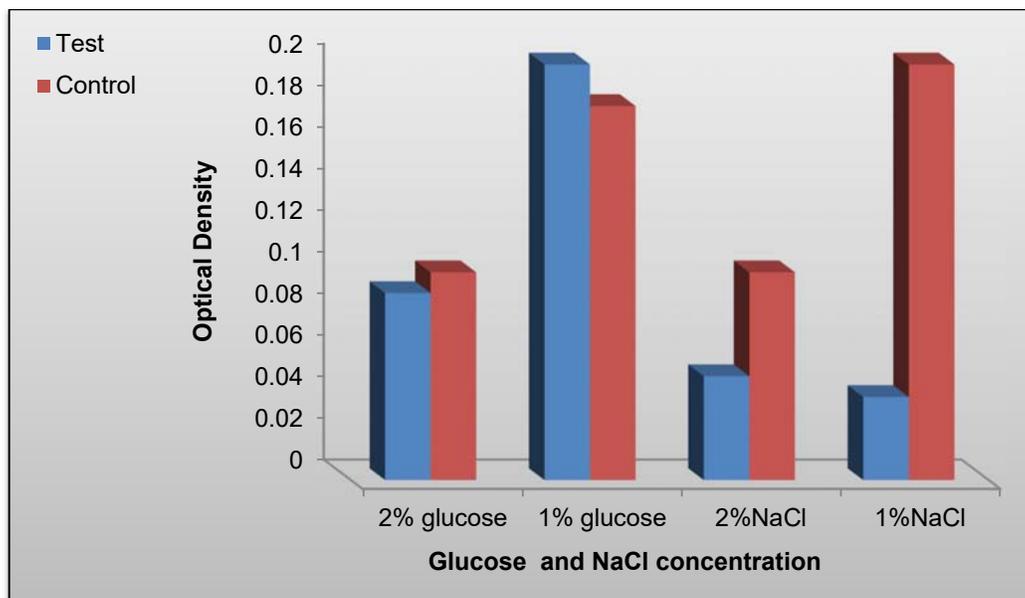


Figure 1: Effect of Glucose and NaCl on the Biofilm Formation of *S. aureus*

Regarding to *P.aeruginosa* isolates, the results in table (6) and figure (2) revealed that glucose sugar, with a concentration of 1%, worked to increase the formation of the biofilm by 80%, while the concentration of 2% inhibited the formation of the biofilm by 20% and reduced the ability of isolates to the formation of the moderate biofilm by 20%, while it increased the ability to form a weak biofilm to 60% after it was 20% at a concentration of 1%, In respect to the effect of sodium chloride, it resulted in the strong biofilm formation by 80% and 100% at a concentration of (1,2%).The relative quantities of extracellular polysaccharide intracellular adhesion and teichoic acids relied on the selection of medium and other growth conditions. Particularly the existence of sugars seemed to play an important role in the stimulation of this progression [19].

Table 6: Effect of Glucose and Sodium Chloride on Biofilm formation of *Pseudomonas aeruginosa* after 24 Hours of Incubation

Number of <i>P. aeruginosa</i> isolates (%)				
Media use	Non biofilm	Weak biofilm	Moderate biofilm	Strong biofilm
NB without enhancer	0 (0%)	25(100%)	0 (0%)	0 (0%)
NB + 1% glucose	0 (0%)	5(20%)	20(80%)	0 (0%)
NB + 2% glucose	5(20%)	15(60%)	5(20%)	0 (0%)
NB + 1% NaCl	0 (0%)	0 (0%)	5(20%)	20(80%)
NB + 2% NaCl	0 (0%)	0 (0%)	0 (0%)	25(100%)

NB; nutrient broth

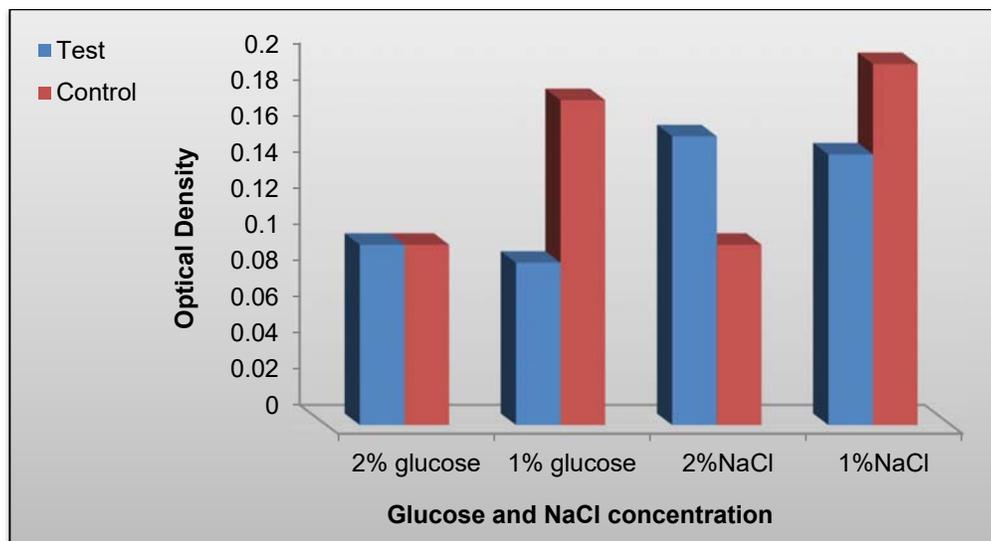


Figure 2: Effect of Glucose and NaCl on the Biofilm Formation of *P.aeruginosa*

4. Conclusions

Supplementing media with 1% glucose led to enhance the biofilm forming capacity while sodium chloride caused inhibition and addition of glucose in concentration more than 1% caused inhibition in biofilm production.

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