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Effect of Some Environmental Stress Factors in *Staphylococcus aureus*' Biofilm

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The purpose of this research was to investigate if *S.aureus* work the formation of biofilms and also to evaluate the impacts of environmental factors on the formation of biofilms. It was shown that the pathogen can form biofilms, which have a negative impact on the health of the organism, leading to resistance to environmental stress factors. This study was designed by use Microtitre-plate(MP) technique for examination of biofilm formation of *S.aureus* and their relationship with some environmental stress factors, which were included temperature, glucose and sodium nitrate. The effects of biofilm formation were tested with Luria-Bertani broth (LB) supplement varying concentrations of glucose (0.1; 0.5;1.0;2.0;3.0)%, sodium nitrite (0.05;0.1;0.15;0.2)%, as well were incubated the medium at different temperatures (25;37;45)°C, individually. It was observed that the addition of glucose (3.0%) was affected by the ability isolates to adhere to the MP-surface. Glucose was showing a positive effect on the formation of isolates of the biofilm while sodium nitrite was negatively affected. Where the isolates were produced the least amount of biofilm at the concentration (0.2%) sodium nitrite. Interestingly, isolates were given the high rate of formation of the biofilm at high temperature 45°C, which has resulted in their classification among the moderate values in the composition of the biofilm.
KEY WORDS: *Staphylococcus aureus*, biofilm, sodium nitrite, temperature, glucose.

1. INTRODUCTION

Staphylococcus aureus (*S.aureus*) is a gram-positive pathogenic bacterium, the most common diseases in the world. About 20-30% of the general population bears this symbiotic bacterial pathogen which leads to suppurative diseases, acquires drug resistance and causes other medical issues[1,2]. Biofilms are described as microbially based sessile societies characterized by cells irreversibly connected to a foundation[3,4]. They are integrated with a matrix of extracellular polymeric substances (EPS) generated by them and display a modified phenotype in terms of development speed and gene transcription[5]. Key nutrient availability, surface chemotaxis, bacterial motility, surface adhesives, and surfactant existence are some variables that affect the development of biofilms[6].

The formation of biofilms depended on the existence of glucose and sodium chloride with ideal staphylococci development temperature. It is not surprising that at 37°C temperature, bacteria form a more biofilm as this temperature reproduced the physiological conditions necessary for growth[7]. Glucose has been shown to cause the phase of biofilm growth in

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multicellular accumulation. It was shown that elevated osmolarity, stimulates the development of biofilm in *S. aureus*[8]. Also, different environmental circumstances, such as elevated temperature, anaerobiosis, elevated salt, glucose, ethanol, and sub-inhibitory antibiotic levels induce the development of polysaccharide intercellular adhesion (PIA)[9,10]. The researchers were showed that nitrite (-NO_2 not acidified) in *S.aureus* societies suppressed biofilm development. Thus, in planktonic and biofilm environments, -NO_2 can either inhibit bacterial growth or kill such bacteria. The fact that it is not an antibiotic supports the passion for its use. Rather, -NO_2 is a biocide against bacteria that are not capable of enzymatically removing it or its decrease products biofilms[11]. Some researchers used glucose-based trypticase soy broth (TSB) and/or brain-heart infusion (BHI) broth and/or Luria-Bertani broth (LB) with sucrose supplements to evaluate the impact on biofilm phenotype[7,12]. There is a clear need to recognize fresh antimicrobial objectives specific to biofilms. Bacteria in biofilms are inherently more resistant to antimicrobial agents than planktonic for a broad range of reasons, including dispersal restriction, modified metabolic activity, phenotypic and genotypic biofilm cell systems, and severe micro-environmental circumstances observed in biofilms. In biofilms, antimicrobial resistance can also be viewed as a multicellular strategy in which the biofilm cells collectively resist antimicrobial factors that would kill a lone cell. Furthermore, planktonic staphylococci resistant to methicillin, make these biofilm-associated " hard to treat " bacteria, even more, challenging to treat with antimicrobial agents presently accessible[13]. Additionally, biofilm production is recognized as an important virulence factor for bacteria of the genus Staphylococci[14]. The aim of this research was to investigate whether *S.aureus* make the formation of biofilms and also to assess the impact of environmental factors on biofilm formation such as temperature, glucose, and sodium nitrite($NaNO_2$).

2. MATERIALS AND METHODS

2.1. Bacterial strains

Two strains, they are positive biofilm-producing *S. aureus*, were assessed by cultured onto Congo Red Agar (CRA). Both bacterial strains were obtained and maintained in TSB, in which 20% glycerol was added, at -80°C from the collection of the laboratories Central Public Health Laboratory in Baghdad. Both isolates were confirmed as *S. aureus* by the conventional microbiological methods and VITCK2 system also.

2.2. Phenotypical definition of ability to produce slime

Production of slime was studied by growth both of *S.aureus* isolates strains on published CRA comprising 0.8g of CR-stain and 50g of sucrose to one litter of BHI-agar[15,16]. Inoculated agar was incubated for 24h at 37°C.

2.3. Quantitative biofilm formation assay using Microtitre plate.

Biofilm formation by the isolates was quantitatively assessed by crystal violet (CV) MP-assay[17,18]. Briefly, individual of *S.aureus* isolate was grown in LB-broth at 37°C for 18 hours at the end of the incubation period; the bacterial culture was diluted in the LB-broth-1% glucose and adjusted of measurement by DensiCHEKplus at 0.5, then 200 µl was

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stabilized in wells of a sterile 96-well polystyrene-MP and was incubated under fixed conditions at 37°C for different times (24;48;72) hours. After incubation, the contents of the wells were taken away from separation, the wells were washed with phosphate buffer saline (PBS), PH 7, four times the removal of unattached cells by using ELISA-washer. The adhesive bacteria were fixed by 100 µl sodium acetate 2% and 100 µl CV-stain solution 1% (w/v) was added per well, vibration the plate three times to aid the blue color to achieve the well 's bottom and incubated at room temperature for 15 min., then CV-dye a solution was completely eliminated from wells by aspiration and washed five times with PBS. Only the cohered bacteria forming the biofilm were held on the surface of the well. After allowing wells to air-raid for 45 min, 200 µl of 95% ethanol was added to each well for 15 min., the contents of the wells were shocked to dissolve CV-dye. The formation of biofilm in the well was measured using at OD630 in each well by using micro-ELISA. All the assays were performed with three replicates for each isolate.

All strains were classified into the following groups: non-adherent(0), weakly(+), moderately(++), or strongly(+++) adhesive, based upon the ODt of bacterial biofilms. Strains were classified as follows: ODt ≤ ODc non-adhesive; ODc <ODt ≤ 2xODc weakly adhesive; 2xODc < ODt ≤ 4xODc moderately adhesive; 4xODc < ODt strongly adhesive. ODt was indicated to the tests were achieved by CV, which obligation to wells exposed to the culture media with bacteria. ODc was indicated to the controls were performed with CV, which obligation to wells exposed only to the culture media without bacteria.

2.3.1. Temperature.

This assay was performed in the same way from above with some improvements, that were incubated at different temperature values (25;37;45)°C at different times (24;48;72) hours.

2.3.2. Concentrations of glucose.

This assay was performed in the same way from above with also some improvements, that was different concentrations of glucose (0.1; 0.5;1.0;2.0;3.0)% posteriorly, incubated at 37 °C for different times (24;48;72) hours.

2.3.3. Concentrations of $NaNO_2$.

This assay was performed in the same way from above, with $NaNO_2$ was added to the broth at different concentrations(0.05;0.1;0.15;0.2)% before entering autoclave posteriorly, incubated at 37°C for different times (24;48;72) hours.

3. RESULTS

Two strains were characterization and biochemical identification were done. Both strains colonies appeared mannitol fermentation, positive for coagulase, production of catalase and ribonuclease enzyme. To confirm a diagnosis, the Vitek2 device was used. The diagnosis rate was 96% *S.aureus*.

The CRA assay style described in the literature were evaluated[12,14]. Sucrose supplement to BHI-media resulted, all colonies on BHI-agar with CR-stain was presented mostly purple to black colonies.

The biofilm formation ability of strains at different temperatures (Table-1).The temperatures of (25;37;45)°C had positive effects on biofilm formation ability of *S.aureus*. In general, nose-related *S.aureus* strains appeared weakly rates biofilm producer

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comparative of ODc rate under all different temperatures after (24;48)hrs, and nose-related *S.aureus* strains were appeared moderately rates biofilm producer under 45°C after 72 hr. All the different temperatures were evaluated significantly affect the biofilm formation in *S.aureus* strains; the results were statistically significant ($p< 0.05$).

Table1-The values ODt- ODc of formation biofilm with different temperatures and time.

Temperature	staph.1 after 24h	staph.1 after 48h	staph.1 after 72h	staph.2 after 24h	staph.2 after 48h	staph.2 after 72h
25°C	0.028(+)	0.037(+)	0.021(+)	0.014(+)	0.044 (+)	0.016(+)
37°C	0.039(+)	0.041(+)	0.030(+)	0.039(+)	0.047(+)	0.039(+)
45°C	0.041(+)	0.048(+)	0.101(++)	0.050(+)	0.052(+)	0.113(++)

non-adherent (0), weakly (+), moderately (++), strongly (+++) adhesive

The addition of glucose in a range from (0.1 to 3.0)% in LB was affecting the ability of the *S.aureus* strains to adhere to the MP,(Table-2) slight increase in the rates of strains weak biofilm producer was detected in increase glucose concentrations; however, the results were statistically significant ($p<0.05$). The optimal glucose concentration for biofilm formation was (3.0%) after 72hr incubated, where the highest rate of weak biofilm formed was observed.

Table 2- The values ODt-ODc of formation biofilm with glucose concentration and time.

Concentration of glucose	staph1 after 24h	staph1 after 48h	staph1 after 72h	staph2 after 24h	staph2 after 48h	staph2 after 72h
0.1% (w/v)	0.009(+)	0.008(+)	0.019 (+)	0.031(+)	0.010(+)	0.010(+)
0.5% (w/v)	0.005(+)	0.026(+)	0.025(+)	0.045(+)	0.022(+)	0.016(+)
1 % (w/v)	0.021(+)	0.027(+)	0.035(+)	0.039(+)	0.033(+)	0.032(+)
2 % (w/v)	0.067(+)	0.030(+)	0.037(+)	0.045(+)	0.043(+)	0.034(+)
3% (w/v)	0.025(+)	0.035(+)	0.051(+)	0.045(+)	0.047(+)	0.066(+)

The influence of $NaNO_2$ concentration has a negative effect on biofilm production was observed for *S.aureus* strains. The presence of 0.2% of $NaNO_2$ in LB displayed an inhibitory effect on the biofilm production to *S.aureus* strains (Table-3) when a decrease in the frequency of weak producers was observed with the times ($p<0.05$).

Table 3- The values ODt- ODc of formation biofilm with $NaNO_2$ concentration and time.

Concentration of NaNO2	staph1 after 24h	staph1 after 48h	staph1 after 72h	staph2 after 24h	staph2 after 48h	staph2 after 72h
0.05%(w/v)	0.033(+)	0.040(+)	0.035(+)	0.044(+)	0.068(+)	0.043(+)
0.1 %(w/v)	0.025(+)	0.023(+)	0.089(+)	0.041(+)	0.034(+)	0.038(+)
0.15%(w/v)	0.023(+)	0.019(+)	0.024(+)	0.037(+)	0.028(+)	0.027(+)
0.2 %(w/v)	0.014(+)	0.000(0)	0.000(0)	0.024(+)	0.000(0)	0.000(0)

non-adherent (0), weakly (+), moderately (++), or strongly (+++) adhesive

4. DISCUSSION

The aim of this study was to determine if there is a distinction in the formation of biofilms between *S.aureus* strains below a range of conditions and to evaluate the impacts of environmental factors on the formation of biofilms. It was interesting to note that under all circumstances, *S.aureus* strains were considerably more frequently listed as low biofilm formers.

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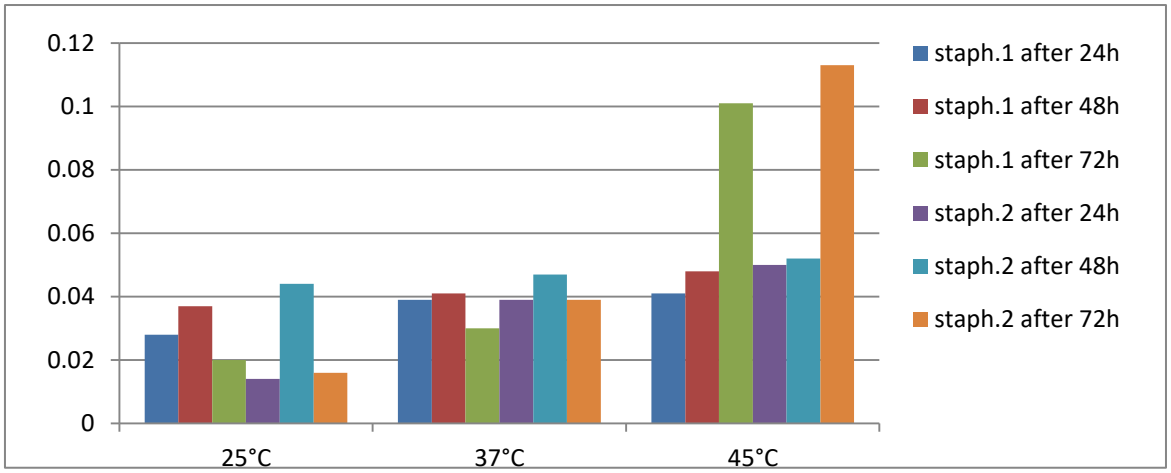

Figure 1- The relationship between temperature,time and biofilm formation in *S.aureus.*

S.aureus was displayed different behavior under different supplementation and temperatures. In the present study, the addition of glucose (3.0%) and temperatures (45°C) after 72hr from incubated in LB increased the power of nose-related *S. aureus* strains to adhere to the MP (Fig-1 & Fig-2). While the addition of $NaNO_2$ (0.2%) after 48h and 72hr from incubated in LB inhibited the power of *S.aureus* strains to adhere to the MP (Fig-3).

In contrast, Koreňová et al. were reported that the biofilms produced by *S.aureus* were moderated after 24 hours of incubation at 37°C [18]. The one research was shown that biofilm is active at temperatures ranging from +5°C to 45°C. In addition to accelerating biofilm formation and growth, elevated temperatures during the original development of biofilms also affect the bio-electrocatalytic efficiency of these biofilms when they were assessed at the same operating temperatures. For example, the time required for the formation of biofilms was reduced from more than 40 days at 15°C to 3.5 days at 35°C [19].

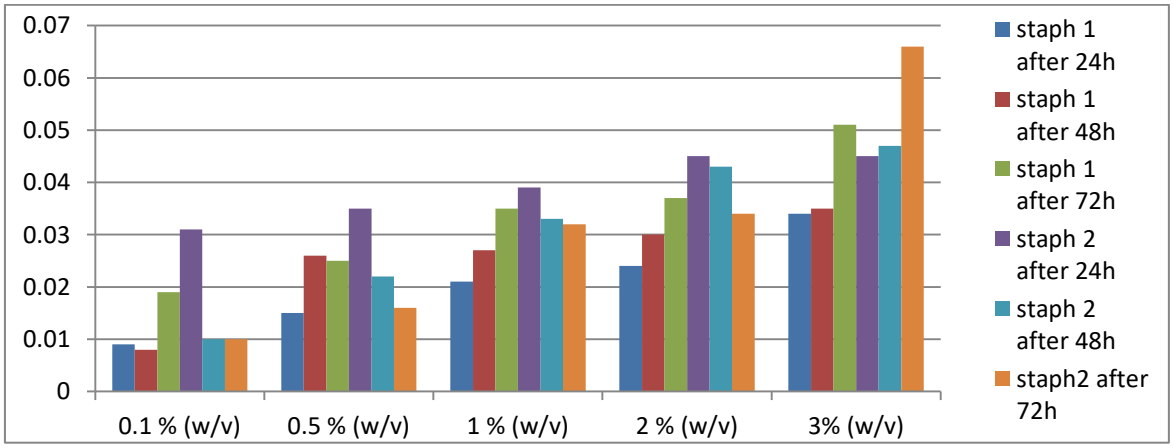

Figure 2- The relationship between glucose concentration, time and formation biofilm in *S.aureus.*

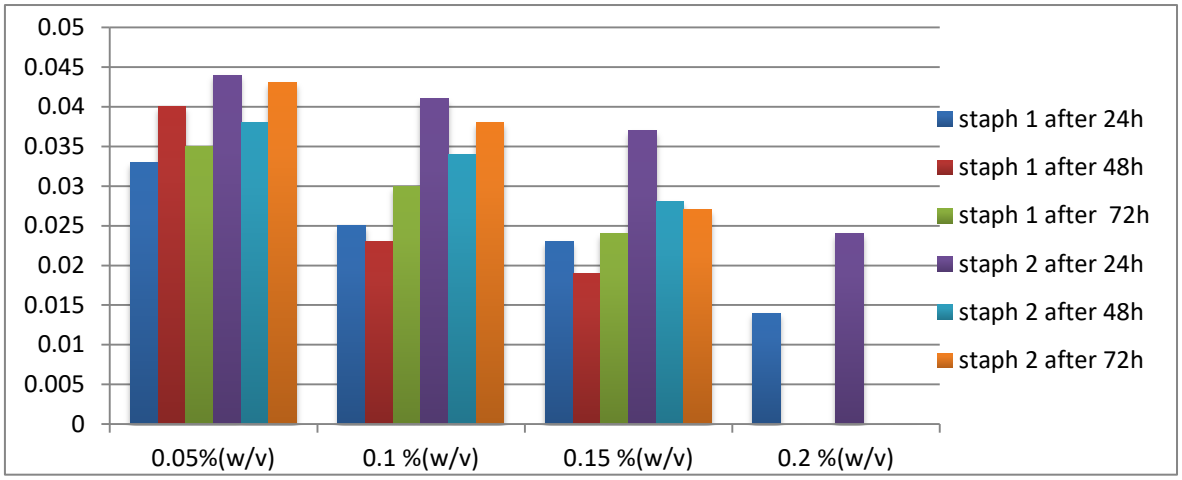

Figure 3- The relationship between $NaNO_2$ concentration, time and formation biofilm in *S.aureus.*

Biofilms produced at high temperatures are more active electrochemical than those produced at reduced incubation temperatures [20]. In this study, it was observed that higher temperatures (37°C compared to 25°C) were responsible for increasing the biofilm production in *S. aureus*, but the high temperatures (45°C) were responsible for maximum increase the biofilm production in *S. aureus*; speculating that this is due to its pathogenicity and difference to the temperature of the human nose. In unlike one of the studies which It was found that lower temperatures (30°C compared from 37°C and 42°C) were responsible for increasing the production of biofilms in *S. epidermidis*, thinking that this was due to its proximity to human skin temperature (33°C) in those countries [13]. The anther study showed that greater temperatures did not influence the biofilm matrix's significant material, but reduced the sessile cell membrane fluidity [21]. Glucose has been specified to influence the formation of in vitro biofilm PIA in *S.aureus* [9, 10]. Nitrite appears to be a jumbled boon for the cells. On the one side, it can play a growth-promoting role; on the other side, elevated levels of nitrite and nitrite-derived compounds can be poisonous, nitrite can produce nitrous acid under acidic circumstances, with powerful bactericidal activity [22]. The final, based on the findings in this study were concluded that the various variables including glucose and temperature in a defined ratio significantly enhances *S.aureus'* biofilm-forming ability of the proposed in vitro biofilm-forming assay using MP. While $NaNO_2$ adversely affects the formation of biofilms with high concentrations. The current research presents a uniform assay for biofilm synthesis by *S.aureus* in vitro MP specimens and its categorization showing their capacity to generate biofilm differently slightly. So the suggested in vitro technique may be further estimated for its utility in the management of continual infections caused by the bacteria.

5. Statistical Analysis.

All experiments are stated as mean ± standard deviation (SD) in triplicate. Statistical and variance analysis (ANOVA) therapies. Major differences were defined as $p< 0.05$ and were based on ANOVA one-way.

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