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STABILITY OF *Staphylococcus aureus* BIOFILM AGAINST DIFFERENT CHEMICAL AGENTS AND SOME ENZYMES

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ABSTRACT

The Biofilm of *Staphylococcus aureus* was tested against a various of substances at different concentrations for 12 hr., to show its stability. The chelating agent Ethylene diamine tetra acetic acid (EDTA) (1-5%) didn't show any effect on the biofilm stability. Oxidizing agents also didn`t show a negative effect on the biofilm stability. By adding enzymes like Pepsin and Papain (100-500µl) the stability of biofilm did not affect. Organic solvents *e.g.*; hexan, toluene and methyl alchohol added at 1-5% also did not destabilize the biofilm stability. Acids and basics like (HCl and NaOH) were added with concentrations of 1-5%. The effect of different substance on the stability of biofilm were very slight. Adjusting the bacterial media to pH 2-13 using HCl and NaOH before the biofilm formation was effected and showed positive effect and prevent biofilm formation between pH (2-5) and pH (10-13).

Key words: *Staphylococcus arues*, biofilm, stability.

INTRODUCTION

Bacterial biofilms are generally described as surface-associated bacterial community forming micro-colonies surrounded by a matrix of exopolymers Microbial biofilms can exist as aggregates more or less confluent, single layer mat or three-dimensional architecture with pors allowing liquid and gas flow and dispersion of nutrients and waste components (Stoodley *et al*., 2002). Any study of bioflms must accept that bioflms may develop in an enormous number of environments, and that the structural intricacies of any single bioflm formed under any specific set of parameters may well be unique to that single environment and microflora. The enormous number of microbial species capable of forming bioflms or interacting with others to do so, together with the very great range of polysaccharides produced, gives rise to an infinite number of permutations. In natural conditions, monospecies bioflms are relatively rare, thus most bioflms are composed of mixtures of micro-organisms.

Bioflms probably comprise the normal environment for most microbial cells in many natural and artificial habitats, and as such are complex associations of cells, extracellular products either trapped within the bioflm or released from cells which have lysed as the bioflm ages (Christensen, 1989). The main `cement' for all these cells and products is the mixture of polysaccharides secreted by the cells established within the bioflm. Probably the nearest analogy is processed food, in which a mixture of macromolecules of all types interact in various ways to form a recognizable structure. Within such a structure, cells, water, ions and soluble low-and high-molecular-mass products are trapped. In many bioflms, as in food, the hydrated polysaccharides may be in a semi-solid state. The major component in the bioflm matrix is water ± up to 97%. Biofilm formation and persistence in estuarine environments is governed by a suite of complex physical, chemical, and biological processes. Many of these parameters can vary significantly over different time scales. For example, nutrient availability can vary over diel light cycles, daily

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tidal cycles, and with rainfall events and seasonal change. Patterns in particulate and dissolved nutrient input to estuarine systems may influence shifts in biofilm bacterial community structure as select guilds respond to their required conditions (Zhang *et al.,* 1998).

Stability of biofilm is a phenomenon that proves the gravity of biofilm formation, In biofilms, individual cells are associated with each other with a matrix, consistsing of exopolysaccharides, proteins, and residue of DNA, and thus are retained in biofilm form under hydrodynamic conditions. Under such conditions, two opposite processes determine the stability of microbial biofilms: cellular attachment and detachment. Both processes are mechanistically linked in that those factors that mediate attachment of cells need to be inactivated when cells detach. Metabolic energy is required for maintenance of biofilm stability. It was previously showed that oxygen depletion or a stop in medium flow, which imposes conditions of oxygen depletion in a hydrodynamic, aerobic biofilm, leads to massive detachment of single cells. Oxygen limitation likely impacts the cellular ATP concentration and that can affect the availability of cellular energy of individual cells and thus the stability of biofilms (Renee *et al.*, 2011).

The usefulness of biofilms is well known, especially in the field of bioremediation. The use of organisms to remove contaminants, *e.g.* metals and radio nuclides, oil spills, nitrogen compounds and for the purification of industrial waste water, is now commonplace. Indeed the adhesive characteristics of natural human flora are now considered as a tool for preventing the adhesion of pathogenic bacteria to avert infection . In the food industry biofilms cause serious engineering problems such as impeding the flow of heat across a surface, increase in fluid frictional resistance of surfaces and increases in the corrosion rate of surfaces leading to energy and production losses. Pathogenic microflora grown on food surfaces and in processing environments can cross-contaminate and cause post-processing contamination. If the microorganisms from food-contact surfaces are not completely removed, they can lead to mature biofilm formation and so increase the biotransfer potential. Examples of the food sectors that pay particular attention to the possibility of cross-contamination are the milk industry and the slaughter industry (Trevor *et al.,* 2008)

MATERIALS AND METHODS

Staphylococcus aureus ATCC6538 used in this study was kindly obtained from Dr. S.Mahgoub, Microbiology Department, Faculty of Agriculture, Zagazig University. The fresh *S. aureus* culture was prepared by inoculating 250 ml of sterilized water with 7.5 g of trypticase soy broth in 500 ml flasks. Organisms isolated from fresh agar plates were inoculated in 10 ml of trypticase soy broth and incubated at 37^{o}C for 24 hr. The cultures were then diluted with fresh media (1:100). Individual wells of sterile 96 well flat bottom polystyrene tissue culture treated plates were filled with 200 µl of the diluted cultures. The plates were incubated at 37^{o}C for 24 hr. After incubation, the non-adhered contents of each well were removed by gentle tapping. The formed biofilm were washed with 0.2 ml of phosphate buffer saline (pH 7.2) four times to remove free floating bacteria, the formed biofilm were fixed by sodium acetate (2%) (Stepanovic *et al.,* 2000). The plates leaved to dry for 1 hr., then stability of the adhered biofilm were tested by adjusting to different chemical agents and enzymes. Every well was filled by 200 µl of each agent and left for 12 hr., at room temperature. The concentration of the reagents were as follows:

1- HCl (1% - 5%)

2- NaOH (1% - 5%)

3- EDTA (1% - 5%)

4- Potassium permanganate (1% - 5%)

5- Potassium dichromate (1% - 5%)

6- Papain Enzyme (100 µl - 500 µl)

7- Pepsine Enzyme (100 µl - 500 µl).

The adhered biofilm stained by crystal violet (0.1%). Excess stain was removed by using deionized water and plates were kept for drying at room temperature . Optical density (OD) of stained adherent biofilm was obtained by using micro ELISA autoreader at wavelength 600 nm.

In another experiment the diluted culture were adjusted to pH 2-13 with adding several volumes of HCl (1N) NaOH (1N) diluted into wells as previously described . The plates were treated as the previously experiment.

RESULTS AND DISCUSSION

Fig. 1 report the effect of EDTA on the stability of *S. aureus* biofilm. Data in this figure show that EDTA didn't generally affect the stability of *S. aureus* biofilm. Slight reduction in biofilm stability can be observed at the whole range of EDTA concentration. The reduction in stability was about 12-13% at 1%, Increasing the concentration didn't shows any further effect.

Data in Fig. 2 show the effect of Oxidizing agents on the stability of *S. aureus* biofilm. The data observe that oxidizing agents didn`t make negative effect on the stability of biofilm. This result is in agreement with those obtained by (Zoran 2012) who investigated the effect of some oxidizing agents like benzalkonium chloride (BAC), sodium hypochlorite (NaClO) chloramines B (CAB), and peracetic acid (PAA) on some biofilm cells of *staphylococcus* spp., *Klebsiella* spp, and *Escherichia coli* and also that attributed the failure of oxidizing agents to affect *S. aureus* biofilm by the format of organic matter.

Data in Fig. 3 show that Pepsin and Papain enzymes have negative effect on the stability of *S. aureus*, These results are in contrast with those obtained by (Tara *et al.*, 2016) who showed that three hydrolytic enzymes are able to degrade biofilm components *i.e.*; amylase, trypsin and lysozyme. The role of these enzymes was investigated in two contexts, removal of biofilms from the environment for food detection and processing and prevention of biofilm formation on the microorganism surface itself. To test this, enzyme activity was measured on feeding appendages of fiddler crabs ,which feed on biofilms, and on egg masses externally brooded by blue crabs. All three enzyme activities were found on feeding appendages of fiddler crabs and in egg masses of blue crabs. For the context of removal of biofilms from the environment. This discrepancy could be due to the difference in the specialty of each enzyme. The failure of Pepsin and Papain to affect the stability of biofilm may be due to the low amount of protein in the biofilm structure.

The results in Fig.4 indicate generally negative effect of HCl and NaOH on the stability of biofilm at a concentration range of (1-5)% since they didn`t have any effect on the stability of Biofilm. Data in Fig.4 indicate the influence of the pH (2-13) on the *S. aureus* biofilm formation. At the acidic media pH (2-5) and the basic range pH (9-13) the biofilm formation was drastically affected, the reduction in the formation of biofilm was at (80-90)% as compared to the biofilm formation at pH 7, Although the high concentration of acidic and basic media did not affected the biofilm stability but slight change in pH value had effects on the biofilm formation. In the same time (Marked *et al.*, 2008) said that changes in pH can have a marked effect on bacterial growth and as such is frequently exploited in the production of detergents and disinfectants used to kill bacteria. Bacteria possess membrane-bound proton pumps which extrude protons from the cytoplasm to enerate a transmembrane electrochemical gradient, *i.e.* the proton motor force. The passive influx of protons in response to the proton motive force can be a problem for cells attempting to regulate their cytoplasmic pH. Large variations in external pH can overwhelm such mechanisms and have a biocidal effect on the microorganisms. Bacteria respond to changes in internal and external pH by adjusting the activity and synthesis of proteins associated with many different cellular processes. Studies have shown that a gradual increase in acidity increases the chances of cell survival in comparison to a sudden increase by rapid addition of HCl. This suggests that bacteria contain mechanisms in place which allow the bacterial population to adapt to small environmental changes in pH. However, there are cellular processes which do not adapt to pH fluctuations so easily. One such process is the excretion of exopolymeric substances (polysaccharides). Optimum pH for polysaccharide production depends on the individual species, but it is around pH 7 for most bacteria.

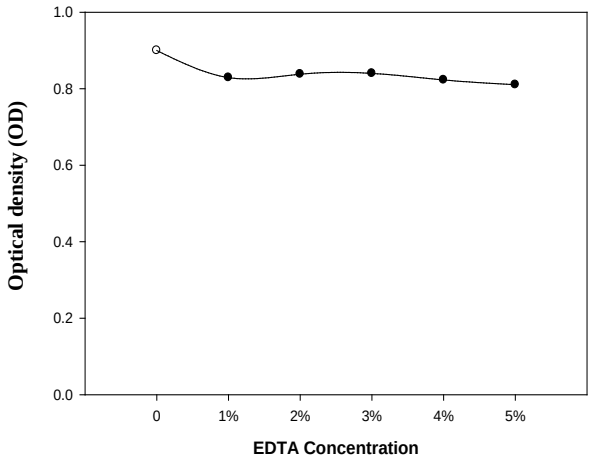

Fig. 1. Effect of Ethylene diamine tetra acetic acid (EDTA) at different concentrations on the stability of *Staphylococcus arues* biofilm

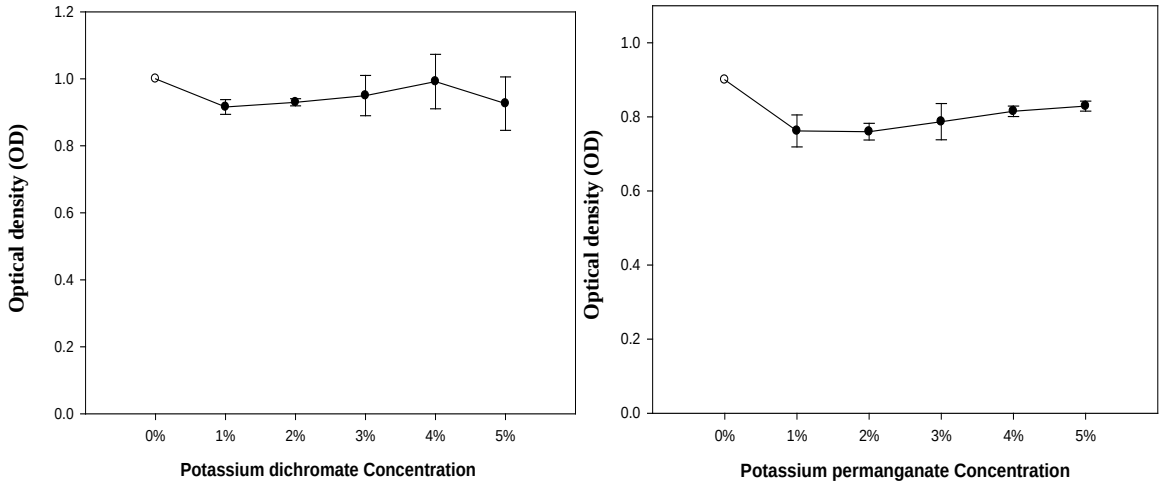

Fig. 2. Effect of Oxidizing agents at different concentrations on the stability of *Staphylococcus arues* biofilm

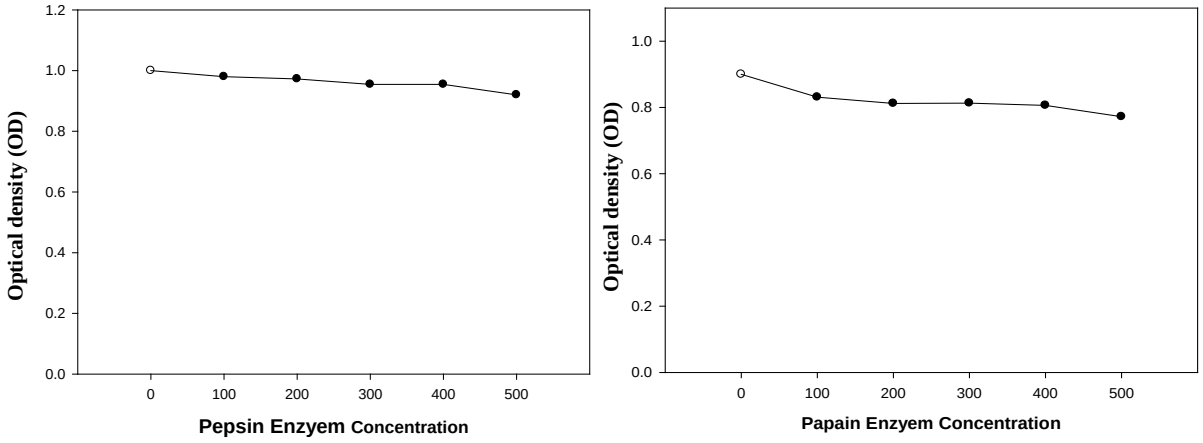

Fig. 3. Effect of Enzyme (Pepsin and Papain) at different concentrations on the stability of *Staphylococcus arues* biofilm

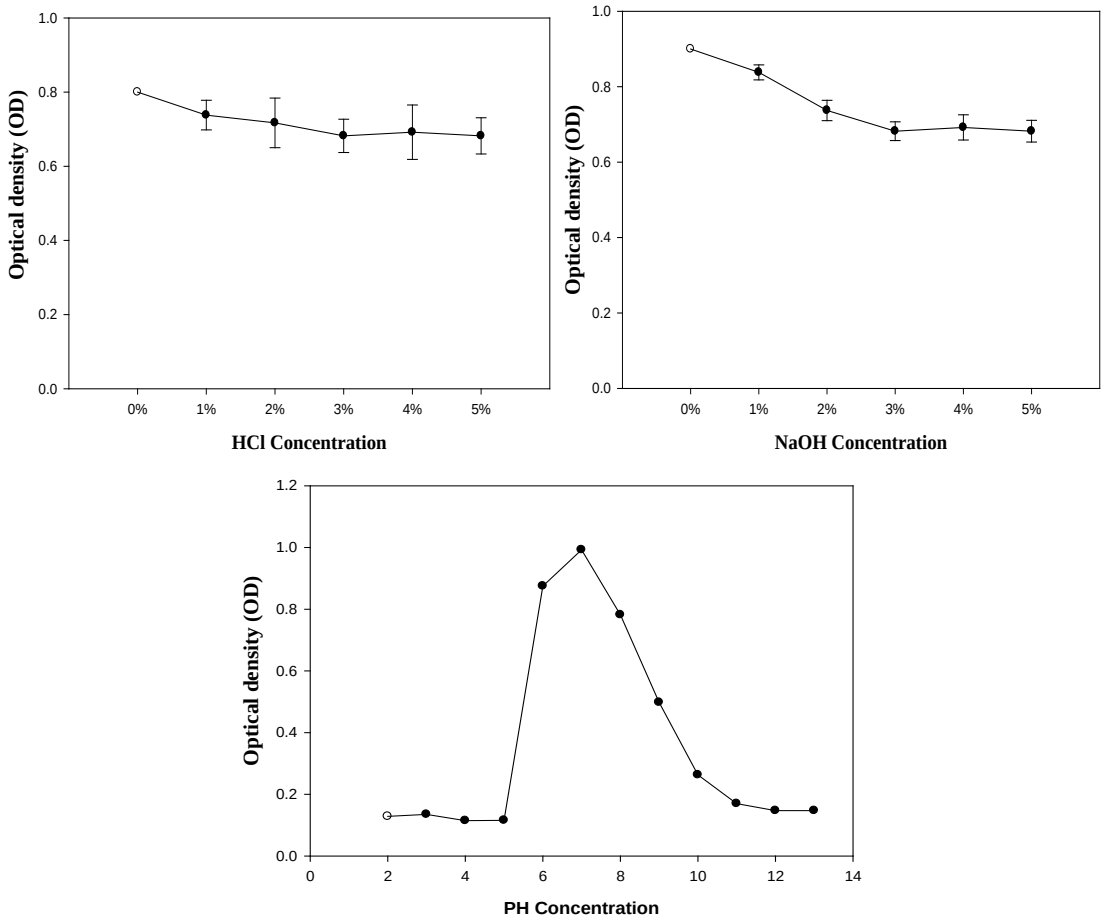

Fig. 4. Effect of Hydrochloric acid and Sodium Hydroxide at different concentrations and pH on the stability and formation of *Staphylococcus arues* biofilm

Conclusion

The results of this study showed that Stability of *Staphylococcus arues* biofilm which considered as a source of free cell of bacteria which very difficult to remove or break and it is better to work on the prevention of biofilm formation in the beginning because the removal of various chemicals or enzymes may be difficult.

REFERENCES

Christensen, B.E. (1989). The role of extracellular polysaccharides in bioflms. J. Biotechnol., 10: 181- 202.

Renee, M.S., S. Rakshe, J.A.J. Haagensen, S. Shukla and A.M. Spormann (2011). Energy-Dependent stability of shewanella oneidensis MR-1 Biofilms, 193 (13): 3257–3264.

Stepanovic, S., D. Vukovic, I. Dakic, B. Savic, and M. Svabic-Vlahovic (2000). A modified microtiter-plate test for quantification of staphylococcal biofilm formation. J. Microbiol. Meth., 40 : 175–179.

Stoodley, P., K. Sauer, D.G. Davies, J.W. Costerton (2002). Biofilms as complex differentiated communities. Annu. Rev. Microbiol., 56:187–209.

Tara, E.B., A. Wepprich, A. Thompson and D. Rittschof (2016). Enzymes manage biofilms on crab surfaces aiding in feeding and antifouling. J. Experim. Marine Biol. and Ecol., 479 : 106–113.

Trevor, R.G., M. Bhakoo and Z. Zhang (2008). Bacterial adhesion and biofilms on surfaces, 18 : 1049–1056.

Zhang, X.Q., P.L. Bishop and M.J. Kupferle (1998). Measurement of polysaccharides and proteins in bioflm extracellular polymers. Water Sci. Technol., 37: 345 - 348.

Zoran, J., D. Červinková, H. Vlková, E. Michu, G. Kunová and V. Babák (2012). Bacterial biofilms resist oxidising agents due to the prese. Organic Matter., 30 (2): 178–187.

ثبات بيوفيلم البكتيريا الكروية العنقودية ضد مختلف العوامل الكيميائية وبعض الإنزيمات

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تم اختبار بيوفيلم لميكروبات العنقودية *Staphylococcus arues* ضد مجموعة متنوعة من المواد في تراكيزات مختلفة لمدة ١٢ ساعة لإظهار مدى استقراره، حيث تم إضافة احد المركبات المخلبية EDTA بتركيز ١-٥% ولكن لم تظهر أي تأثير على ثبات البيوفيلم، تم إضافة عوامل مؤكسدة أيضا والتى أظهرت تأثير سلبي على ثبات البيوفيلم، الإنزيمات مثل البيبسين والبابين أُضيفت بتركيز ١٠٠- ٥٠٠µl ولم يتأثر ثبات البيوفيلم أيضاً، المذيبات العضوية مثل الهكسان والتولوبين وكحول الميثيل أضيفت بتركيز ١-٥% ولم تزعزع ثبات البيوفيلم، تم إضافة الأحماض والقواعد مثل حامض الهيدروكلوريك وهيدروكسيد الصوديوم في تركيزات ١-٥ % وكان تأثيرهم على ثبات البيوفيلم طفيف جداً، ضبط درجة الحموضة بين 2-13 pH باستخدام حمض الهيدروكلوريك وهيدروكسيد الصوديوم قبل أن يتكون البيوفيلم وأظهرت تأثير إيجابي ومنع تشكيل بيوفيلم بين (٢-٥) pH وكذلك من (١٠-١٣) pH.

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