J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 50(2): 409-415, 2026
Doi: 10.33483/jfpau.1748338
Research Article / Araştırma Makalesi

OPTIMIZATION OF *IN-VITRO* BIOFILM FORMATION IN STRAINS OF *STREPTOCOCCUS MUTANS* AND *STAPHYLOCOCCUS EPIDERMIDIS* IN DIFFERENT GROWTH CONDITIONS

STREPTOCOCCUS MUTANS VE STAPHYLOCOCCUS EPİDERMİDİS SUŞLARINDA FARKLI BÜYÜME KOŞULLARINDA İN-VİTRO BİYOFİLM OLUŞUMUNUN OPTİMİZASYONU

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ABSTRACT

Objective: *Microorganisms reside in polymer-coated communities known as biofilms, which represent a major virulence factor by conferring resistance to treatment in associated infections. Biofilms are therefore a frequent subject of health-related research. However, standard laboratory strains have been cultured under inconsistent conditions, with limited data available on optimal biofilm production parameters. This study aimed to determine the optimal in vitro biofilm formation conditions for Streptococcus mutans ATCC 25175 and Staphylococcus epidermidis ATCC 35984 by systematically evaluating growth media, sugar types/concentrations, atmospheric conditions, and incubation periods.*

Material and Method: *S. mutans and S. epidermidis strains were cultured under various conditions, including media (Brain Heart Infusion and Mueller Hinton Broth), sugar types/concentrations (sucrose and glucose), atmospheres (aerobic vs. 5% CO_2), and incubation times (24 and 48 hours). Biofilm production was quantified by standard crystal violet staining in microtiter plates.*

Result and Discussion: *Highest biofilm production for S. mutans occurred in BHI with 2% sucrose, under 5% CO_2, after 24-48 hours. For S. epidermidis, peak biofilm was in MHB with 1% glucose, under aerobic conditions, after 24 hours. These findings highlight the need for species-specific optimization of biofilm-inducing conditions when using standard strains.*

Keywords: *Biofilm, glucose, Staphylococcus epidermidis, Streptococcus mutans, sucrose*

ÖZ

Amaç: *Mikroorganizmalar, polimer kaplı topluluklar olan biyofilmler içinde yaşarlar. Biyofilm, önemli bir virülans faktörü olup neden olduğu enfeksiyonların tedavisi güçtür ve birçok sağlık çalışmasının konusudur. Ancak, bu çalışmalarda kullanılan standart suşların optimum biyofilm üretim koşulları yeterince bilinmemektedir. Bu çalışma, Streptococcus mutans ATCC 25175 ve Staphylococcus epidermidis ATCC 35984 suşlarının optimum biyofilm üretim koşullarını; farklı büyüme ortamları, şeker konsantrasyonları, atmosferik koşullar ve inkübasyon süreleri açısından araştırmayı amaçlamaktadır.*

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Submitted date: 22.07.2025 **Accepted date:** 10.04.2026 **Published date:** 19.05.2026
Citation: Savluk, M., Ünal, N. (2026). Optimization of *in-vitro* biofilm formation in strains of *Streptococcus mutans* and *Staphylococcus epidermidis* in different growth conditions. Journal of Faculty of Pharmacy of Ankara University, 50(2), 409-415.

Gereç ve Yöntem: *S. mutans ve S. epidermidis suşları, farklı kültür ortamları (Brain Heart Infusion – BHI ve Mueller Hinton Broth – MHB), şeker türleri/konsantrasyonları (sükroz ve glukoz), atmosferik koşullar (aerobik ve %5 CO_2) ile inkübasyon süreleri (24 ve 48 saat)'nde in vitro koşullarda kültüre edilerek, biofilm üretimi mikrotiter plakalarda standart kristal viyole boyama yöntemiyle kantitatif olarak ölçülmüştür.*

Sonuç ve Tartışma: *Elde edilen bulgulara göre; S. mutans'ın en yüksek biyofilm üretimi, %2 sükroz ilaveli BHI ortamında, %5 CO_2 atmosferinde ve 24–48 saat inkübasyonda gözlenmiştir. S. epidermidis için ise en yüksek biyofilm üretimi, %1 glukoz içeren MHB ortamında, aerobik koşullarda ve 24 saat inkübasyonda tespit edilmiştir. Bu bulgular, özellikle standart laboratuvar suşlarının kullanıldığı sağlık odaklı araştırmalarda, biyofilm indükleyici koşulların türe özgü optimizasyonunun önemini ortaya koymaktadır.*

Anahtar Kelimeler: *Biyofilm, glukoz, Staphylococcus epidermidis, Streptococcus mutans, sükroz*

INTRODUCTION

Biofilms are surface-attached microbial clusters embedded in an extracellular polymeric matrix, exhibiting spatial and functional heterogeneity. They are widespread not only various environments, but also the human body [1,2]. The exopolysaccharide (EPS) matrix, composed of carbohydrates, proteins, nucleic acids, and cell wall polymers, is essential for biofilm stability and cellular coordination [3]. This structure enhances bacterial survival by evading host defenses, resisting environmental stress, and tolerating antimicrobials [1].

Biofilms significantly contribute to clinical infections, particularly chronic diseases, as multidrug-resistant pathogens reinforce EPS matrix formation [4,5]. Biofilm-embedded bacteria exhibit antibiotic tolerance up to 1000 times higher than their planktonic counterparts [6].

The oral microbiome, the second-largest microbial community in the human body, serves as a reservoir for antibiotic resistance genes. Biofilms are prevalent in dental plaque, with *Streptococcus mutans* and *Staphylococcus epidermidis* as key contributors to infections. *S. mutans* utilizes biofilms for survival on tooth surfaces [7], while *S. epidermidis*, a commensal organism, can cause dental caries, implant infections, and nosocomial infections, by biofilm production as its main virulence factor [8,9].

Biofilm development is influenced by nutrient availability, incubation conditions, and antibiotic exposure [10,11]. It progresses through four stages: attachment, microcolony formation, maturation, and dispersion [2]. Various phenotypic methods, including Congo red agar, tube method, tissue culture plate (TCP) assay, and microscopy, are used for biofilm characterization [12,13].

As no single method ensures optimal biofilm production, this study aims to identify the best conditions for *S. mutans* and *S. epidermidis* by evaluating medium composition, sugar content, incubation atmosphere, and duration.

Different studies employ various conditions [10,11], and no single method guarantees optimal biofilm production. By evaluating medium composition, sugar content, incubation atmosphere, and duration this study aims to determine the best conditions for *S. mutans* and *S. epidermidis* biofilm production.

MATERIAL AND METHOD

Bacterial Strains

Three bacterial strains were used for biofilm production tests: *S. mutans* ATCC® 25175 (KWIK-STIK™ USA), a second *S. mutans* ATCC® 25175 strain, both associated with dental caries, and *S. epidermidis* ATCC® 35984, a known biofilm producer.

Biofilm Production Conditions

Optimal biofilm conditions were assessed using different factors: sucrose (1% and 2%) for *S. mutans*, glucose for *S. epidermidis*, incubation atmospheres (aerobic, 5% CO_2), durations (24, 48 hours), and three nutrient media (TSB, MHB, BHIB; Merck-Germany) (Table 1).

Biofilm Analyses

Biofilm production was evaluated using the microplate test with eighteen different media (BHIB (no sucrose), BHIB (+1% sucrose), BHIB (+2% sucrose), MHB (no sucrose), MHB (+1% sucrose), MHB (+2% sucrose), TSB (no sucrose), TSB (+1% sucrose), TSB (+2% sucrose), BHIB (no glucose), BHIB (+1% glucose), BHIB (+2% glucose), MHB (no glucose), MHB (+1% glucose), MHB (+2% glucose), TSB (no glucose), TSB (+1% glucose), TSB (+2% glucose)). Bacterial suspensions were prepared according to 0.5 McFarland standard ($1x10^8$ CFU/ml) from 24 h pure culture and these suspensions (1/100 dilution) were incubated in microplates under aerobic or 5% CO_2 conditions. After incubation, biofilms were quantified using the tissue culture plate (TCP) method [5]. Wells were stained with 0.1% crystal violet for fifteen minutes, washed three times, treated with 33% glacial acetic acid for fifteen minutes, and measured at 620 nm using a spectrophotometer (BioTek Epoch, BioSPX-Belgium) [12] (Figure 1). All experiments were done three times.

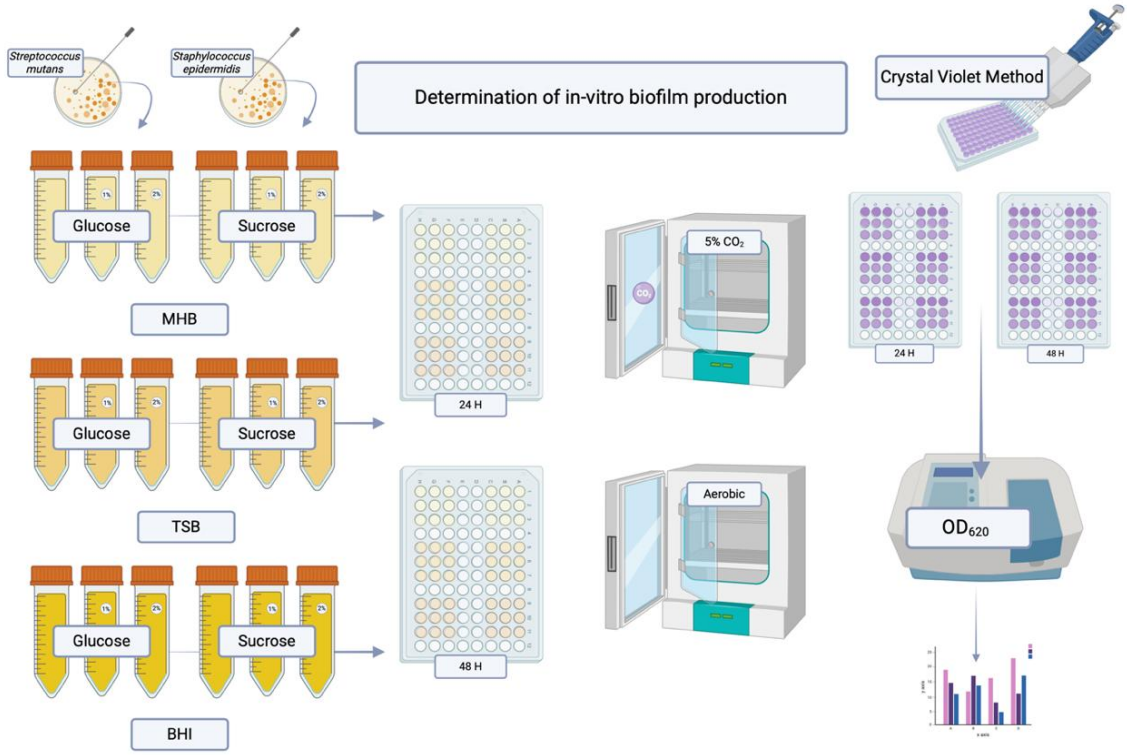

Figure 1. Determination of *in vitro* biofilm production and strain growth

RESULT AND DISCUSSION

Biofilm production characteristics were classified according to average OD values as follows: <0.120 non-producer, 0.120–0.240 moderate, and >0.240 strong biofilm producer [4]. The biofilm production results are summarized in Table 1.

Biofilm production values of *S. mutans* strains, ranged between 0.0643±0.007 and 3.1293±0.57. The highest biofilm production (3.1293±0.57) was observed after 24 hours of incubation in TSB medium supplemented with 2% sucrose under 5% CO_2 conditions. This was closely followed by 2.9090±0.77 in BHIB with 2% sucrose after 48 hours under the same atmosphere. Overall, *S. mutans* was a strong biofilm producer in all atmospheric conditions and all media containing added sugar (Table 1).

Table 1. Biofilm productions

Strains and Media	Incubation Period- Atmosphere Conditions OD_{620}±SD			
	24h		48h	
	Aerobic	5% CO_2	Aerobic	5% CO_2
***S. mutans* ATCC® 25175**				
MHB (no sugar)	0.0643±0.007	0.1313±0.11	0.0770±0.01	0.0773±0.01
MHB (+1% sucrose)	0.4933±0.13	0.4270±0.11	0.5750±0.04	0.5013±0.11
MHB (+2% sucrose)	0.4900±0.04	0.4193±0.05	0.6233±0.07	0.7156±0.04
TSB (no sugar)	0.0966±0.01	0.1173±0.01	0.1066±0.01	0.1213±0.03
TSB (+1% sucrose)	0.9646±0.43	2.5730±0.64	2.3710±0.13	2.6946±0.70
TSB (+2% sucrose)	1.8320±0.45	2.1443±0.50	2.7803±0.49	2.7493±0.46
BHIB (no sugar)	0.0706±0.001	0.0776±0.003	0.0670±0.007	0.0836±0.01
BHIB (+1% sucrose)	2.1290±0.94	1.9776±0.40	2.7590±0.64	2.7830±0.49
BHIB (+2% sucrose)	1.5550±0.22	3.1293±0.57	2.7623±0.54	2.9090±0.77
KWIK-STIK™ *S. mutans* derived from ATCC® 25175™				
MHB (no sugar)	0.0580±0.006	0.0616±0.001	0.0756±0.04	0.0660±0.002
MHB (+1% sucrose)	0.8360±0.14	0.5346±0.06	0.7566±0.08	0.8070±0.04
MHB (+2% sucrose)	0.8323±0.08	0.5626±0.04	0.7346±0.10	0.8063±0.15
TSB (no sugar)	0.1716±0.02	0.2143±0.005	0.2323±0.02	0.3486±0.09
TSB (+1% sucrose)	2.0033±0.45	3.0333±0.46	3.1656±0.17	3.2066±0.29
TSB (+2% sucrose)	2.0656±0.42	3.1953±0.52	3.1153±0.23	3.2283±0.23
BHIB (no sugar)	0.0690±0.009	0.0700±0.008	0.0733±0.01	0.0713±0.009
BHIB (+1% sucrose)	2.8686±0.54	3.3010±0.34	3.2713±0.21	3.0263±0.20
BHIB (+2% sucrose)	3.1133±0.61	3.0330±0.44	3.500±0.00	3.2543±0.28
***S. epidermidis* ATCC® 35984**				
MHB (no sugar)	2.1293±0.50	0.2420±0.03	2.3516±0.05	0.4313±0.03
MHB (+1% glucose)	2.4510±0.51	0.3850±0.01	1.8166±0.11	0.3766±0.02
MHB (+2% glucose)	2.4276±0.188	0.4283±0.15	1.7263±0.06	0.4326±0.08
TSB (no sugar)	1.6606±0.85	0.0633±0.006	1.3580±0.32	0.1780±0.07
TSB (+1% glucose)	2.1173±1.16	0.1133±0.04	1.8020±0.12	0.0903±0.01
TSB (+2% glucose)	3.0993±0.20	0.1870±0.08	1.5436±0.48	0.1063±0.02
BHIB (no sugar)	0.0816±0.01	0.0566±0.01	1.0126±0.12	0.2936±0.06
BHIB (+1% glucose)	0.9673±0.86	0.0820±0.02	0.9693±0.36	0.0953±0.01
BHIB (+ 2% glucose)	0.7363±1.04	0.1036±0.03	1.0853±0.74	0.0976±0.001

Biofilm production values of the KWIK-STIK™ *S. mutans* ATCC® 25175™ strain ranged from 0.0580±0.006 to 3.500±0.00. The highest biofilm production (3.500±0.00) occurred in BHIB supplemented with 2% sucrose under aerobic conditions after 48 hours of incubation, followed by anaerobic biofilm production (3.2543±0.28) in the same medium and incubation period. Biofilm production values in BHIB with 2% sucrose remained consistently high, ranging from 3.0330±0.44 to 3.500±0.00 (Table 1).

Biofilm production values of *S. epidermidis,* varied between 0.0566±0.01 and 3.0993±0.20 across different media, incubation times, and atmospheric conditions. The highest biofilm production (3.0993±0.20) was recorded in TSB medium with 2% glucose after 24 hours in aerobic conditions, followed by MHB with 1% glucose (2.4510±0.51) under the same conditions. *S. epidermidis* was a strong biofilm producer in all MHB media tested (Table 1).

The purpose of this study was to explore the optimal biofilm production conditions of *S. mutans* and *S. epidermidis* ATCC strains, both known as biofilm formers and commonly used in biofilm assay. Nine different media with sugar concentrations of 0%, 1%, and 2% (sucrose for *S. mutans* and glucose for *S. epidermidis*) were prepared, and bacteria were incubated under aerobic and 5% CO_2 conditions for 24 and 48 hours.

Nutrient availability is a critical factor influencing bacterial biofilm production. Several previous studies have used TSB for biofilm quantification [13,14]. A recent study also employed TSB for biofilm production in multiple bacterial species, including *S. mutans* and *S. epidermidis*. Based on previous lab experience and literature, sucrose was used instead of glucose for *S. mutans*. Two *S. mutans* and one *S. epidermidis* strain, one from our laboratory culture collection and one purchased from KWIK, were used in the study. Comparing biofilm production between TSB and BHIB, opposite trends were observed for *S. mutans* and *S. epidermidis* from the culture collection; biofilm production reached maximum levels, particularly in *S. mutans* from the KWIK brand cultured with BHIB and sucrose. This indicates possible strain-dependent differences in biofilm production even under identical media and incubation conditions.

Bacterial adhesion to plates showed a linear increase over time in all media, particularly for both *S. mutans* strains. Similarly, Efimenko et al. [5] reported increased biofilm production after 48 hours in et-peptone medium using the TCP method. Shamsulddin et al. [2] described *S. mutans* strains as moderate to strong biofilm producers when incubated in TSB with 1% glucose under 5% CO_2 conditions for 72 hours. Our strains were strong biofilm producers at all incubation times in TSB with 1% sucrose under 5% CO_2 conditions.

The KWIK strain and the culture collection strain *S. mutans* showed good and moderate biofilm production under different conditions. However, minor differences in biofilm production between these strains appeared depending on environmental conditions and incubation times (Table 1), suggesting phenotypic variation related to storage conditions and passage numbers.

The optimal growth medium used for *S. mutans* biofilm production was BHIB supplemented with 2% sucrose. BHIB is rich in leucine, proline, serine, and aspartate, amino acids important for synthesizing adhesins such as fibronectin-binding protein and aggregation factors necessary for adhesion [1,3]. Moreover, *S. mutans* uniquely metabolizes sucrose enzymatically into extracellular polysaccharides via glucan synthases, forming an adhesive matrix that facilitates bacterial aggregation and robust biofilm production. Lipids such as choline and sphingosine in BHIB may further enhance biofilm production and resistance to desiccation [2].

The highest biofilm production of *S. epidermidis* was observed after 24 hours in TSB with 2% glucose under aerobic conditions. Biofilm production increased with glucose addition in all media. Moderate biofilm production occurred in TSB under 5% CO_2, while no biofilm was detected in other media. Although the highest OD value for *S. epidermidis* (3.0993±0.20) was in aerobic TSB with 2% glucose at 24 hours, MHB with 1% glucose under aerobic conditions also supported high biofilm levels (2.4510±0.51) at both 24 and 48 hours. Thus, TSB was the best growth medium, followed by MHB, and aerobic conditions favored biofilm production. Biofilm production peaked at 24 hours and decreased at 48 hours, indicating that incubation time and medium composition affect biofilm dynamics.

Tang et al. [8] used BHIB with 0.5% glucose biofilm production of *S. epidermidis*, while this study used BHIB with 1% and 2% glucose in this study was used. Even low sugar levels enhance biofilm production.

In this study, biofilm formed in both TSB and BHIB media by *S. epidermidis* under aerobic conditions however, it was not formed in BHIB medium with glucose under 5% CO_2. These findings underscore the importance of atmospheric conditions for biofilm production. Consistent with our results, Asai et al. [10] reported decreased biofilm production at elevated CO_2 pressures, and Stepanovic et al. [15] found reduced biofilm production in CO_2 environments.

Consequently, the best growth medium for *S. epidermidis* is TSB, the optimal atmosphere is aerobic, the best sugar concentration is 2% glucose, and the ideal incubation time is 24 hours. However, when conditions vary (incubation time, sugar concentration, atmosphere), MHB supports sustained biofilm production, suggesting it contains major biofilm-enhancing components. The best growth medium for the two *S. mutans* strains is BHIB, with an optimal 5% CO2 atmosphere, an optimal sugar

concentration of 2% sucrose, and an ideal incubation time of 24–48 hours. However, minor differences in biofilm production were observed between the *S. mutans* KWIK strain and the culture collection strain, depending on environmental conditions and incubation times. The findings of the present study can shed light on the importance of species-specific optimization of biofilm-inducing conditions in experimental setups, especially when using standard laboratory strains in health-related research for future.

AUTHOR CONTRIBUTIONS

Concept: M.S., N.Ü.; Design: M.S., N.Ü.; Control: N.Ü.; Sources: M.S., N.Ü.; Materials: M.S., N.Ü.; Data Collection and/or Processing: M.S.; Analysis and/or Interpretation: M.S.; Literature Review: M.S., N.Ü.; Manuscript Writing: M.S., N.Ü.; Critical Review: M.S., N.Ü.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

REFERENCES

1. Shakya, P., Nayak, A., Sharma, R.K., et al. (2022). Effect of incubation time on the biofilm formation by methicillin resistant *Staphylococcus aureus*. Indian Journal of Animal Research, 59(7), 1220-1223. [CrossRef]
2. Shamsulddin, D.Z., Bunyan, A.I. (2024). Isolation and identification of *Streptococcus* mutans and study of biofilm formation. Thi-Qar Medical Journal, 27(1), 255-262.
3. Jakubovics, N.S., Goodman, S.D., Mashburn-Warren, L., et al. (2021). The dental plaque biofilm matrix. Periodontology 2000, 86(1), 32-56. [CrossRef]
4. Manandhar, S., Singh, A., Varma, A., et al. (2018). Evaluation of methods to detect *in vitro* biofilm formation by staphylococcal clinical isolates. BMC Research Notes, 11(1), 714. [CrossRef]
5. Efimenko, A., Ishchenko, O., Stepanskyi, O., et al. (2023). Microbiological features of *Staphylococcus* associated with complications of dental implantation. Wiadomosci Lekarskie, 76(5 Pt 1), 936-943. [CrossRef]
6. Jonblat, S., As-Sadi, F., Zibara, K., et al. (2024). *Staphylococcus* epidermidis biofilm assembly and self-dispersion: bacteria and matrix dynamics. International Microbiology, 27(3), 831-844. [CrossRef]
7. Shemesh, M., Tam, A., Steinberg, D. (2007). Expression of biofilm-associated genes of *Streptococcus* mutans in response to glucose and sucrose. Journal of Medical Microbiology, 56(Pt 11), 1528-1535. [CrossRef]
8. Tang, B., Gong, T., Cui, Y., et al. (2020). Characteristics of oral methicillin-resistant *Staphylococcus* epidermidis isolated from dental plaque. International Journal of Oral Science, 12(1), 15. [CrossRef]
9. Divakar, D.D., Alfawaz, A.S., AlKheraif, A.A., et al. (2017). High proportions of *Staphylococcus* epidermidis in dental caries harbor multiple classes of antibiotics resistance, significantly increase inflammatory interleukins in dental pulps. Microbial Pathogenesis, 109, 29-34. [CrossRef]
10. Asai, K., Yamada, K., Yagi, T., et al. (2015). Effect of incubation atmosphere on the production and composition of staphylococcal biofilms. Journal of Infection and Chemotherapy, 21(1), 55-61. [CrossRef]
11. Dursun, A.D., Uçak, S., Yavuz, O., et al. (2022). Determination of the effect of glucose, sucrose and sodium chloride addition in different culture media on biofilm formation of methicillin resistant *Staphylococcus aureus*. Anatolian Current Medical Journal, 4(2), 152-157. [CrossRef]
12. Christensen, G.D., Simpson, W.A., Younger, J.J., et al. (1985). Adherence of coagulase-negative staphylococci to plastic tissue culture plates: A quantitative model for the adherence of staphylococci to medical devices. Journal of Clinical Microbiology, 22(6), 996-1006. [CrossRef]
13. Knobloch, J.K., Horstkotte, M.A., Rohde, H., et al. (2002). Evaluation of different detection methods of biofilm formation in *Staphylococcus aureus*. Medical Microbiology and Immunology, 191(2), 101-106. [CrossRef]
14. Singh, A.K., Prakash, P., Achra, A., et al. (2017). Standardization and classification of *in vitro* biofilm formation by clinical isolates of *Staphylococcus aureus*. Journal of Global Infectious Diseases, 9(3), 93-

101. [CrossRef]
15. Stepanovic, S., Djukic, N., Djordjevic, V., et al. (2003). Influence of the incubation atmosphere on the production of biofilm by staphylococci. Clinical Microbiology and Infection, 9(9), 955-958. [CrossRef]