ECRONICON OPEN ACCESS

EC MICROBIOLOGY
Research Article

Effect of Growth Media and Optical Density on Biofilm Formation by *Staphylococcus epidermidis*

Mohammad Abdulraheem AL-kafaween[1]*, Abu Bakar Mohd Hilmi[1], Rao Sanaullah Khan[2] and Mabrouka Bouacha[3]

[1]*Faculty of Health Sciences, University Sultan Zainal Abidin, Terengganu, Malaysia*

[2]*School of Food and Agricultural Sciences, University of Management and Technology, Lahore, Pakistan*

[3]*Laboratory of Biochemistry and Microbiology, Department of Biochemistry, Faculty of Sciences, University of Badji Mokhtar, Annaba, Algeria*

***Corresponding Author:** Mohammad Abdulraheem AL-kafaween, Faculty of Health Sciences, University Sultan Zainal Abidin, Terengganu, Malaysia.

Received: October 26, 2018; **Published:** March 25, 2019

Abstract

Objective: The purpose of the present study was to assess the effects of media, optical density, and incubation time on biofilm formation by *Staphylococcus epidermidis.*

Methods: The main procedure involved was fixing the bacterial film with 95% ethanol, staining with 0.1% crystal violet, releasing the bound dye with 33% glacial acetic acid, and measuring the optical density (OD) of the solution at 590 nm by using a microplate reader.

Results: It was found that 3 and 5 days of incubation are critical for biofilm formation as indicated by the OD values of 0.55 - 0.06 and 0.70 - 0.39 in MHB and TSB respectively at OD 0.05. Similar results were noted for OD 0.1 in both media MHB and TSB while TSB showing slightly better results for biofilm formation.

Conclusion: It is concluded that three days (72 hours) are required for obtaining effective biofilm formation in both MHB and TSB at 370C at OD 0.05 and OD 0.1.

Keywords: *Biofilm; Staphylococcus epidermidis; Mueller-Hinton Broth; Tryptic Soy Broth; Optical Density*

Introduction

Staphylococcus epidermidis (*S. epidermidis*) is a Gram-positive, predominantly human skin flora, considered as the normal human flora [1,2]. Recently it has been discovered that *S. epidermidis,* previously considered non-pathogenic, is one of the major pathogens in nosocomial infections [3,4]. Additionally, *S. epidermidis* is a major cause of infections especially in prosthetic joint implants i.e., medical device associated infections [5]. The absence of non-invasive treatment options for joint pathology, surgical interventions become necessary for joint infections including replacement surgery [6]. It has been argued that the ability of *S. epidermidis* to form a strong adherence to implanted biomedical devices is considered its strong virulence factor [5]. Genomic characterization of the species revealed a well-equipped ability to protect itself under hard conditions in its natural habitat [2,4]. Furthermore, *S. epidermidis* has a very powerful osmoprotection system with eight sodium ion/proton exchangers and six transport systems under severe salt concentrations [2]. From the medical care and food safety and hygiene point of view, the bacteria's ability to form a biofilm on plastic surfaces is considered a major virulence factor via quorum sensing mechanism [4,7,8]. Quorum sensing (QS) refer to the differential expression of genes that help in establishing intercellular communication among the bacteria leading to better resistance against antibiotics [8]. Biofilm formation is a complex process comprising of two steps i.e. adhesion and aggregation. First step is the adhesion to the extracellular polysaccharide modified or coated by the host, while the second step involves the aggregation of multilayered bacterial cells [3,4,7,9,10].

A difference in osmotic pressure of the media will have its impact on the growth and biofilm formation. Therefore the effect of media on biofilm formation has been investigated in this model study. A comparison of Mueller Hinton broth (MHB) and Tryptic Soya broth (TSB) has been experimented to understand biofilm formation pattern by *S. epidermidis*. Mueller Hinton broth (MHB) with beef infusion and casein acid hydrolysate provide nitrogenous compounds, carbon, sulphur, and other essential nutrients. Starchacts to provide protective colloid against toxic substances present in the medium. Starch hydrolysis yields dextrose, which serves as a source of energy [11]. MHB formulation was originally developed as a simple, transparent agar medium for the cultivation of many pathogenic bacteria [12]. MHB is now also widely used for biofilm formation by *S. pyogenes* and *Pseudomonas aeruginosa* [13]. MHB is recommended to be diluted for antimicrobial susceptibility testing of all species; most commonly encountered aerobic and facultative anaerobic bacteria. While Tryptic Soya broth (TSB) is recommended as a general purpose medium for the isolation and cultivation of a wide variety of bacteria [5,14,15]. Soyabean casein digest medium is recommended by various pharmacopeias as a sterility testing and as a microbial limit testing medium [16,17]. The combination of pancreatic digest of casein and papain digest of soybean meal makes the medium nutritious by providing amino acids and long-chain peptides for the growth of microorganisms [17,18]. Dextrose and dibasic potassium phosphate serve as the carbohydrate source and the buffer, respectively in the medium. Sodium chloride maintains the osmotic balance of the medium.

Based on the above-mentioned properties of both the media, biofilm formation has been compared in Mueller-Hinton Broth (MHB) and Tryptic Soy Broth (TSB) with the following hypothesis.

- H_1: Effect of media has no significant effect on biofilm formation.
- H_2: Incubation time (days) has no significant effect on biofilm formation.
- H_3: Interaction of media and incubation time has no significant effect on biofilm formation.

Materials and Methods

Bacterial growth

A Gram-positive *S. epidermidis* was used for biofilms formation obtained from the microbiology laboratory of the Faculty of Medicine in University Sultan Zainal Abidin. *S. epidermidis* inoculums were prepared by selecting two morphologically identical colonies from the stock culture followed by suspending them into 5 mL of sterile MHB and TSB into sterilized bottles. The inoculums were incubated at 37°C for 24 hours [1,9,19].

Biofilm Assay

A 2 ml of the inoculum was removed aseptically from the universal bottle and poured into a micro-cuvette (Fisher Scientific, UK). The turbidity of the bacteria was measured in optical densities at the 600nm with a spectrophotometer and adjusted to OD 0.05 and 0.1 after 24 hours incubation in MHB and TSB. Subsequently, *S. epidermidis* (OD 0.05 and OD 0.1) biofilms were prepared by transferring 100 µl of adjusted inoculums into sterile 96 wells plates (Fisher Scientific, UK). The sterile broth was used as a negative control. The plates were incubated at 37°C for 3, 5 and 7 days. After the incubation period, the media were removed by slightly tapping the plates. The wells were washed three times with sterile water to remove free-floating planktonic bacteria then were drained off by inverting to allow air dry. The plates were stained with 150 µl 0.2% (w/v) crystal violet dye for detecting the biofilms. The stained wells were washed three times with Phosphate buffered saline (PBS). After washing, 150 µl of 90% ethanol was used to detach the biofilms from the wells. The solubilized biofilm formations were measured by microplate reader at the wavelength of 590 nm [18,20]. The experiments were performed in triplicate.

Biofilm analysis

In order to classify biofilm formation into various categories, three standard deviations above the mean OD of the negative control (OD_c) was considered to the cut-off value [18]. The following criteria were used to classify the different adherent strength: $OD \leq OD_C$ = non-adherent, $ODC < OD \leq (2 \times OD_C)$ = weakly adherent; $(2 \times OD_C) < OD \leq (4 \times OD_C)$ = moderately adherent and $(4 \times OD_C) < OD$ = strongly adherent [18,21,22].

Statistical analysis

All the trials were conducted in triplicate to calculate the mean and standard deviations of the data collected. An in-depth statistical analysis was followed up by the Two Way ANOVA for determining the factors effective in biofilm formation using Graph Pad Prism 7.04 Software [18,23]. The incubation periods (days/hours) and optical densities were also compared; the mean difference was considered significant at $p < 0.05$.

Results and Discussion

It was found that higher OD values were recorded for TSB (OD 0.1) during the first three days of incubation more than in MHB (OD 0.1) (Figure 1). TSB (OD 0.05 and OD 0.1) was found to be more effective than MHB (OD 0.05 and OD 0.1) under similar conditions of temperature and initial turbidity [21]. It was noticed that after the fifth day of incubation OD values decreased markedly in both of the media.

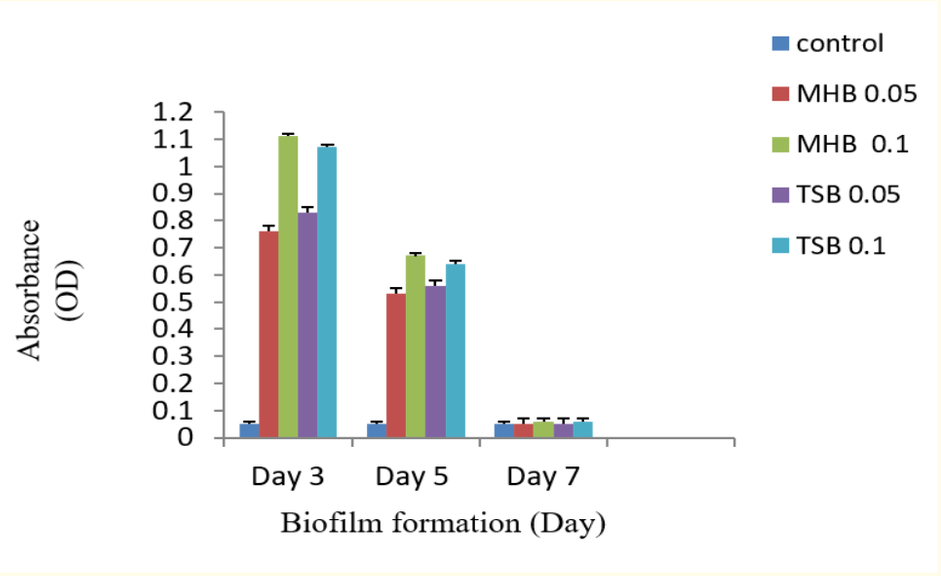

Figure 1: *Biofilm formation by S. epidermidis in MHB and TSB media.*

Further, it was noticed that OD 0.05 and 0.1, in both MHB and TSB, had similar biofilm formation progress (Figure 1) with day 3 and day 5 being strongly adherent ($(4 \times OD_c) < OD$) (Table 1). However, it was also observed that TSB is more effective at OD 0.05 and 0.1 than MHB. It has been reported that the cell membrane of the bacteria grown in TSB is bit more fluid than MHB [24]. This may have an effect on biofilm formation pattern in both the media.

Biofilm characterization

Based upon the OD values, biofilm was characterized into various categories such as strongly adherent, weakly adherent and no adherence (Table 1). It was found that there was a strong adherence at three days of incubation at both OD 0.05 and 0.10 for both media (MHB and TSB).

Times (day)	Control	MHB 0.05	MHB 0.1	TSB 0.05	TSB 0.1
Day 3	0.05	0.76 SA	1.11 SA	0.83 SA	1.07 SA
Day 5	0.05	0.53 SA	0.67 SA	0.56 SA	0.64 SA
Day 7	0.05	0.05 WA	0.06 WA	0.05 WA	0.06 SA

Table 1: *Characterization of biofilms based upon OD values.*

Note: $OD \leq OD_c$ = Non-Adherent, $ODC < OD \leq (2 \times OD_c)$ = Weakly Adherent (WA); $(2 \times OD_c) < OD \leq (4 \times OD_c)$ = Moderately Adherent (MA) and $(4 \times OD_c) < OD$ = Strongly Adherent (SA).

To further explore the relationship, if any, between OD values and incubation time, Two Way ANOVA with repeated measures was performed (Table 2). It was found that OD has a non-significant effect on biofilm formation (P = 0.2167, P > 0.05). Therefore, null hypothesis H_1 is accepted. However, the days of incubation had a significant effect on biofilm formation (P < 0.05); reject the second hypothesis. It was also confirmed that the interaction of media type and days of incubation had no significant effect on biofilm formation (H_3).

Factors	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.1226	2	0.06128	F (2,4) = 3.181	P = 0.1490
Time	0.5074	2	0.2537	F (2,4) = 13.17	P = 0.0174*
Media	0.1689	1	0.1689	F (1, 2) = 3.175	P = 0.2167
Residual	0.07704	4	0.01926		

Table 2: *Two Way ANOVA for determining critical factors in biofilm formation.*

*SS: Sum of Squares; DF: Degree of Freedom; MS: Mean Square; F (DFn, DFd): F Distribution; *P < 0.05 considered significant.*

Turkey's multiple comparison tests were also performed to check the significance of time (days) on biofilm formation in both media. It was found that the time (days) had a significant effect (P < 0.05) on biofilm formation (Table 1) under both 0.05 and 0.10 OD. To further explore the critical time for biofilm formation Turkey's multiple comparisons were performed (Table 2).

Turkey's Multiple Comparison	Mean 1	Mean 2	Mean Diff.	SE of diff.	Adjusted P Values
Day 3 vs. Day 5	0.5531	0.3075	0.2456	0.09814	0.1357
Day 3 vs. Day 7	0.5531	0.04948	0.5036	0.09814	*0.0149
Day 5 vs. Day 7	0.3075	0.04948	0.258	0.09814	0.1196

Table 3: *Two way ANOVA with Turkey's multiple comparison tests.*

**: Significant.*

It was noticed that three-five days (72 - 120 hours) of incubation at 37°C is the ideal time for biofilm formation in MHB and TSB for *S. epidermidis*. Incubation time has been critical as found in other similar studies [5].

Conclusion

It can be concluded that *Staphylococcus epidermidis* performed little better in TSB than in MHB under a similar condition of time and OD. Further, it was also found that incubation time of 72 hours (three days) is optimum for biofilm formation. Initial OD 0.05 and 0.1 has little effect on biofilm formation pattern. However, these are very preliminary qualitative findings which need to be further quantified and biochemically analyzed.

Bibliography

1. Nakatsuji T., *et al*. "A Commensal Strain of Staphylococcus Epidermidis Protects against Skin Neoplasia". *Science Advances* 4.2 (2018).

2. Otto Michael. "Staphylococcus Epidermidis - the Accidental Pathogen". *Nature Reviews Microbiology* 7.8 (2009): 555-567.

3. Rupp ME., *et al*. "Characterization of the Importance of Staphylococcus Epidermidis Autolysin and Polysaccharide Intercellular Adhesin in the Pathogenesis of Intravascular Catheter-Associated Infection in a Rat Model". *Journal of Infectious Diseases* 183.7 (2001): 1038-1042.

4. Zhang YQ., *et al*. "Genome-Based Analysis of Virulence Genes in a Non-Biofilm-Forming Staphylococcus Epidermidis Strain (Atcc 12228)". *Molecular Microbiology* 49.6 (2003): 1577-1593.

5. Stepanovic S., *et al*. "Quantification of Biofilm in Microtiter Plates: Overview of Testing Conditions and Practical Recommendations for Assessment of Biofilm Production by Staphylococci". *Apmis* 115.8 (2007): 891-899.

6. Büttner Henning., *et al*. "Structural Basis of Staphylococcus Epidermidis Biofilm Formation: Mechanisms and Molecular Interactions". *Frontiers in Cellular and Infection Microbiology* 5 (2015): 14.

7. Hussain M., *et al*. "A 140-Kilodalton Extracellular Protein Is Essential for the Accumulation of Staphylococcus Epidermidis Strains on Surfaces". *Infection and Immunity* 65.2 (1997): 519-524.

8. Nandi Sisir. "Recent Advances in Ligand and Structure Based Screening of Potent Quorum Sensing Inhibitors against Antibiotic Resistance Induced Bacterial Virulence". *Recent Patents on Biotechnology* 10.2 (2016): 195-216.

9. Cucarella C., *et al*. "Bap, a Staphylococcus Aureus Surface Protein Involved in Biofilm Formation". *Journal of Bacteriology* 183.9 (2001): 2888-2896.

10. Speziale P., *et al*. "Protein-Based Biofilm Matrices in Staphylococci". *Frontiers in Cellular and Infection Microbiology* 4 (2014): 171.

11. Murray Patrick R and Ellen Jo Baron. "Manual of Clinical Microbiology". Washington, D.C.: ASM Press (2003).

12. Mueller J Howard and Jane Hinton. "A Protein-Free Medium for Primary Isolation of the Gonococcus and Meningococcus". *Experimental Biology and Medicine* 48.1 (1941): 330-333.

13. Dalhoff Axel., *et al*. "Comparative in Vitro Activities of the Novel Antibacterial Finafloxacin against Selected Gram-Positive and Gram-Negative Bacteria Tested in Mueller-Hinton Broth and Synthetic Urine". *Antimicrobial Agents and Chemotherapy* 55.4 (2011): 1814-1818.

14. Hall M., *et al*. "Comparison of Two Liquid Blood Culture Media Containing Sodium Polyanethole Sulfonate: Tryptic Soy and Columbia". *Applied Microbiology* 27.4 (1974): 699-702.

15. Vijayakumari Siji Jinachandrannair., *et al*. "Optimization of Media and Temperature for Enhanced Antimicrobial Production by Bacteria Associated with Rhabditis Sp". *Iranian Journal of Microbiology* 5.2 (2013): 136-141.

16. Schau HP and JF Macfaddin. "Media for Isolation - Cultivation - Identification - Maintenance of Medical Bacteria". Volume I. Williams and Wilkins (1985).

17. Singh R., *et al*. "Penetration of Antibiotics through Staphylococcus Aureus and Staphylococcus Epidermidis Biofilms". *Journal of Antimicrobial Chemotherapy* 65.9 (2010): 1955-1958.

18. Nyenje ME., *et al*. "Evaluation of the Effect of Different Growth Media and Temperature on the Suitability of Biofilm Formation by Enterobacter Cloacae Strains Isolated from Food Samples in South Africa". *Molecules* 18.8 (2013): 9582-9593.

19. Nuryastuti T., *et al*. "Effect of Cinnamon Oil on Icaa Expression and Biofilm Formation by Staphylococcus Epidermidis". *Applied and Environmental Microbiology* 75.21 (2009): 6850-6855.

20. Jaffar N., *et al*. "Mature Biofilm Degradation by Potential Probiotics: Aggregatibacter Actinomycetemcomitans Versus Lactobacillus Spp". *PLoS One* 11.7 (2016): e0159466.

21. Stepanovic S., *et al*. "A Modified Microtiter-Plate Test for Quantification of Staphylococcal Biofilm Formation". *Journal of Microbiological Methods* 40.2 (2000): 175-179.

22. Basson A., *et al*. "Evaluation of Adherence, Hydrophobicity, Aggregation, and Biofilm Development of Flavobacterium Johnsoniae-Like Isolates". *Microbial Ecology* 55.1 (2008): 1-14.

23. Nyenje Mirriam E., *et al*. "Biofilm Formation and Adherence Characteristics of Listeria Ivanovii Strains Isolated from Ready-to-Eat Foods in Alice, South Africa". *The Scientific World Journal* (2012): 873909.

24. Johnson Seth Russell. "The Effect of Media Selection on the Membrane Characteristics and Fatty Acid Composition of Staphylococcus Aureus". Illinois State University (2013).

Volume 15 Issue 4 April 2019