RESEARCH ARTICLE

Evaluation of the effect of different growth media and incubation time on the suitability of biofilm formation by *Pseudomonas aeruginosa* and *Streptococcus pyogenes*

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Abstract: Introduction: Biofilm formation has different stages and can be classified based on the bacterial strain, culture vessel, and the method employed. Biofilm formation is carried out in culture vessels to represent mode of infection in humans. Microbial concentration, growth medium, supplement, and incubation time are key factors to successfully form biofilm in a culture vessel. **Objective:** This study aimed to identify the optimum conditions for biofilm formation in a 96-well plate by culturing *Pseudomonas aeruginosa* and *Streptococcus pyogenes*. **Materials and Methods:** We utilized the infectious and pathogenic bacteria, *P. aeruginosa* and *S. pyogenes* strains. These bacteria were cultured in Mueller-Hinton Broth (MHB) and Tryptic Soy Broth (TSB) at two different optical densities (OD 0.05 and OD 0.1). After a certain incubation time, the formed biofilm was stained by using 0.1% crystal violet. The stained bacteria were disaggregated and measured using a microplate reader. Biofilm was then classified based on bacterial adherence to the plate. **Results:** Our results showed that *P. aeruginosa* and *S. pyogenes* biofilms were strongly formed on days 3 and 5 in MHB and TSB, respectively. However, the strongest biofilm formation was seen on day 3 after *P. aeruginosa* being incubated in MHB at OD 0.1 and after *S. pyogenes* being incubated in MHB at OD 0.05. **Discussion:** Biofilm formation is ranged between weak, moderate, and strong in accordance with the density of bacterial adhesion. **Conclusion:** *P. aeruginosa* and *S. pyogenes* biofilms were optimized at specific OD (0.1 and 0.05, respectively) for 3 days cultivation in MHB.

Keywords: Biofilm, Mueller-Hinton Broth (MHB), Tryptic Soy Broth (TSB), *Pseudomonas aeruginosa*, *Streptococcus pyogenes*

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1 Introduction

Bacteria commonly remain in a self-produced polymeric matrix and adhere to an inert or living surface (Jain et al., 2013). Microenvironment community of thousands of bacteria is known as the biofilm (Mohammad et al., 2020; Jadhav and Tale, 2015). Biofilm contains the colonized bacteria embedded in the amorphous extracellular material and is usually visualized as a thin layer, which is a unique feature to distinguish the free-living planktonic cells (Chua et al., 2014; Mohammad et al., 2021). The biofilm formation depends on multiple factors including mechanical aspects, organic chemistry, and the genome of the microorganism (Mohammad et al., 2020; Nyenje et al., 2013). Biofilm plays a vital role in the etiopathology of the diseases providing antibiotic resistance (Hφiby et al., 2011). Anatomical and physiological features of bacteria support biofilm formation, e.g., flagella, fimbriae, and pili are crucial in developing a bridge between the bacteria and substrate. The downstream product of biofilm is the hydrated matrix extracellular polymeric substance (EPS) consisting of proteins, polyuronic acids, nucleic acids, and lipids (Al-Bakri and Mahmoud, 2019; Mohammad et al., 2020; Mangwani et al., 2012). Biofilm attachment, detachment, mechanical strength, antibiotic resistance, and exoenzymatic degradation activity are dependent on EPS (Tarawneh et al., 2021; Winkelstrter et al., 2014; Yang et al., 2011).

Streptococcus pyogenes is a species of facultative, gram-positive bacteria made up of nonsporing, nonmotile cocci called group A streptococci (GAS) (Stevens, 2014). *S. pyogenes* is the most pathogenic bacteria and clinically important for human illness (Johansson et al., 2010). *S. pyogenes* is commensal in the nasopharynx of healthy individuals but is responsible for a wide range of diseases including ear infection, skin infection, acute streptococcal sore throat, scarlet fever, and puerperal fever (Tadesse and Alem, 2006). Reports

suggest that the persisting existence of streptococci within human cells may develop resistance against host immune defense system and antibiotic treatment (Rohde and Cleary, 2016). Although penicillin, erythromycin, and tetracycline treatment failure have been reported, antibiotics are still preferred for streptococcal infections (Kataja et al., 2002), (Magnussen et al., 2016). Thus, further study is required to eradicate streptococcal infection. *Pseudomonas aeruginosa* is a common aerobic, gram-negative, and rod-shaped bacterium that belongs to the Pseudomonadaceae family (Mohammad et al., 2021; Palanisamy et al., 2014). It can persist within a wide range of temperatures between 37°C and 42°C (Wolska, 2008). Being a free-living organism, it is commonly found in soil, vegetation, water, marshes, and coastal marine habitats. About 4%-12% of the human population possess *P. aeruginosa* in the gastrointestinal tract (GIT) (Sharma et al., 2014). *P. aeruginosa* produces a variety of infectious pigments such as pyoverdin (yellow-green), pyorubin (red-brown), and pyocyanin (blue-green) (El-Fouly et al., 2015). Reducing its infection is a crucial aim in clinical setting.

Mueller-Hinton Broth (MHB) is an antimicrobial susceptibility testing medium composed of beef, dehydrated infusion from casein hydrolysate, and starch. MHB was initially developed as transparent agar and used as a cultivation medium for many species of pathogenic bacteria (Mueller and Hinton, 1941). It is widely used for biofilm formation by *P. aeruginosa*, *S. pyogenes*, and many other infectious microorganisms. MHB is recommended for antimicrobial susceptibility testing of most commonly encountered aerobic and facultative anaerobic bacteria (National Committee for Clinical Laboratory Standards, 2000). Tryptic Soy Broth (TSB) is recommended for the isolation and cultivation of various aerobic and facultative anaerobic bacteria. Soya bean casein digest medium is another name for TSB and is recommended by various pharmacopeias as sterility and microbial limit testing medium (MacFaddin, 1985). This medium is a highly nutritious and contains a digest of casein and peptic to provide amino acids and long-chain peptides for the growth of microorganisms. Dextrose and dibasic potassium phosphate serve as the buffer (carbohydrate source), whereas sodium chloride provides the osmotic balance for TSB. In human diseases, pathogenic bacteria, such as *P. aeruginosa* and *S. pyogenes*, are the initial colonizers in all main organs including tooth. Their interaction with the substrate facilitates the first biofilm formation as a plaque (Yadav and Prakash, 2017). Plaque formation requires pellicle formation and reversible adhesion that involves weak, long-range physicochemical interactions (Young et al., 2016). Interaction between cell surface-pellicle and higher adhesion-receptor mediates the biofilm attachment and adhesion among cells (Longo et al., 2014). *P. aeruginosa* and *S. pyogenes* biofilms are the primary infection in human illness. Therefore, we aimed to establish the formation of *P. aeruginosa* and *S. pyogenes* biofilms in vitro for future degradation and inhibition experiments.

2 Materials and Methods

2.1 Bacterial growth

P. aeruginosa (ATCC 10145) and *S. pyogenes* (ATCC 19615) purchased from Teraslab Saintifik (Malaysia) were used for biofilm formation. *P. aeruginosa* and *S. pyogenes* inoculum were prepared from stock culture by selecting three to five morphologically identical colonies and were suspended into 4-5 mL of sterile MHB and TSB in sterilized universal bottles. The inoculum was incubated at 37°C for 24 hours (Shehu et al., 2016), (Jibril et al., 2019), (Fatima et al., 2018).

2.2 Biofilm assay

A few single colonies were suspended in sterile MHB and TSB before incubation at 37°C for 24 hours. After incubation, the suspension of MHB and TSB was adjusted to OD 0.1 and 0.05 for each broth, respectively. Then, 200 μL of adjusted bacterial suspension was transferred to microtiter plate. The negative control contained 200 μL of broth without bacteria. *P. aeruginosa* and *S. pyogenes* biofilms were observed for their adherence to microtiter plate after 3, 5, and 7 days of incubation. At the end of incubation, the solution was discarded, and the well was washed three times with 200 μL of phosphate-buffered saline to remove planktonic bacteria. The plates were air-dried and stained with 200 μL of 1% crystal violet for 10 min. The plates were carefully rinsed under running tap water to remove excess stain, air-dried at room temperature before solubilizing the biofilm with 200 μL of 95% ethanol. Biofilms were characterized using a microplate reader at 570 nm (Tecan Infinite 200 PRO, Austria). The experiments were performed in triplicate (Mohammad et al., 2021; Jaffar et al., 2016; Mohammad et al., 2020; Tarawneh et al., 2021).

2.3 Biofilms analyses

The following criteria were used to classify the different adherent strengths: strong adherent [SA = (4 × ODC) < OD)], moderate adherent [MA = (2 × ODC) < OD ≤ (4 × ODC)], weak adherent [WA = ODC < OD ≤ (2 × ODC)], or nonadherent [NA = OD ≤ ODC)]. ODC was referred to the density of the negative control (Al-kafaween et al., 2019; Al-kafaween et al., 2019a; Mohammad et al., 2021; Nyenje et al., 2013).

2.4 Statistical analyses

Quantitative analysis of biofilm formation was performed by using SPSS (version 20.0: IBM). Mean differences were determined by using an independent t-test. Statistical significance was set at $p < 0.05$.

3 Results

Figure 1 demonstrates the reproducibility of *P. aeruginosa* biofilm. On day 3, the biofilms strongly adhered to the plate after incubation with MHB = 0.76±0.11, MHB OD 0.1 = 1.11 ±0.01, TSB OD 0.05 = 0.83 0.0±, and TSB OD 0.1 = 1.07 ±0.02. On day 5, the biofilms strongly adhered to the well; MHB OD 0.05 = 0.53 ±0.05, MHB OD 0.1 = 0.67 ± 0.04, TSB OD 0.05 = 0.56 ± 0.05, and TSB OD 0.1 = 0.64 ± 0.09. However, the adherence was lesser than the biofilms on day 3. On day 7, biofilms did not adhere to the well after incubation with MHB OD 0.05 = 0.05 ± 0.0, MHB OD 0.1 = 0.06 ± 0.01, TSB OD 0.05 = 0.05 ± 0.0, and TSB OD 0.1 = 0.06 ± 0.01.

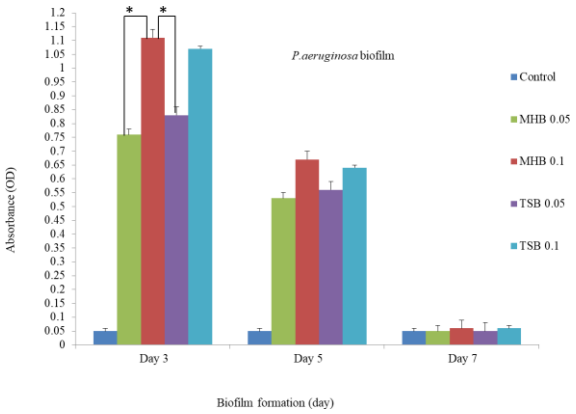

Figure 1. Effects of growth medium and optical density on biofilm formation by *P. aeruginosa* in MHB (OD 0.05 and 0.1) and TSB (OD 0.05 and 0.1). Asterisk (*) indicates that independent t-test was significant ($p < 0.05$).

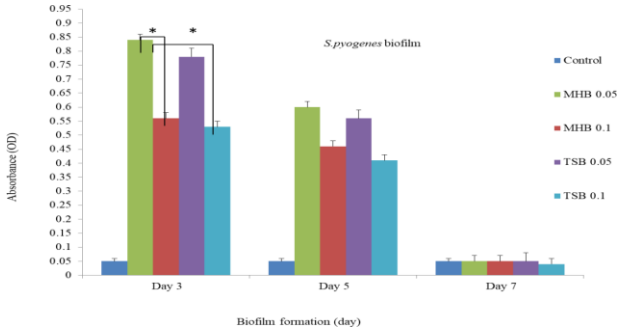

Figure 2. Effects of growth medium and optical density on biofilm formation by *S. pyogenes* in MHB (OD 0.05 and 0.1) and TSB (OD 0.05 and 0.1). Asterisk (*) indicates that independent t-test was significant ($p < 0.05$).

Figure 2 demonstrates the reproducibility of *S. pyogenes* biofilm. On day 3, biofilms strongly adhered to the plate after incubation with MHB OD 0.05 = 0.84 ± 0.05, MHB OD 0.1 = 0.56 ± 0.07, TSB OD 0.05 = 0.78 ± 0.07, and TSB OD 0.1 = 0.53± 0.02. On day 5, the biofilms strongly adhered to the well; MHB OD = 0.6 ± 0.02, MHB OD 0.1 = 0.46 ± 0.03, TSB OD 0.05 = 0.56 ± 0.05, and TSB OD = 0.41 ± 0.02. However, the adherence was lesser than biofilms on day 3. On day 7, biofilms did not adhere to the well after incubation with MHB OD 0.05 = 0.05 ± 0.01, MHB OD 0.1 = 0.05 ± 0.0, TSB OD 0.05 = 0.05 ± 0.01, and TSB OD 0.1 = 0.04 ± 0.0.

Table 1 and Table 2 demonstrates the classification of biofilm formation by *P. aeruginosa* and *S. pyogenes* on mi-

Table 1. Classification of *P. aeruginosa* biofilms

Medium/ Initial inoculum concentration	Control	Day 3	Day 5	Day 7	*P* -value
Parameters	Mean ODc± SD	Mean OD ± SD	Mean OD ± SD	Mean OD ± SD	MHB OD_{600} (0.05) compare with TSB OD_{600} (0.05) **No significance (0.089)**
MHB OD_{600} (0.05)	0.05±0.01	0.76±0.11 **(SA)**	0.53 ± 0.05 **(SA)**	0.05 ± 0.0 **(NA)**	MHB OD_{600} (0.05) compare with TSB OD_{600} (0.1) （**0.013**）
MHB OD_{600} (0.1)	0.05±0.01	1.11±0.01 **(SA)**	0.67 ± 0.04 **(SA)**	0.06 ± 0.01 **(NA)**	MHB OD_{600} (0.1) compare with TSB OD_{600} (0.05) **(0.016)**
TSB OD_{600} (0.05)	0.05±0.01	0.83±0.07 **(SA)**	0.56 ± 0.05 **(SA)**	0.05 ±0.0 **(NA)**	MHB OD_{600} (0.1) compare with TSB OD_{600} (0.1) **No significance (0.110)**
TSB OD_{600} (0.1)	0.05±0.01	1.07±0.02 **(SA)**	0.64 ± 0.09 **(SA)**	0.06 ± 0.01 **(NA)**	TSB OD_{600} (0.1) compare with TSB OD_{600} (0.05) **(0.018)**
					MHB OD_{600} (0.05) compare with MHB OD_{600} (0.1) **(0.014)**

Note: Mueller Hinton Broth (MHB), Tryptic Soy Broth (TSB), Non-adherent (NA), Weakly adherent (WA), Moderately adherent (MA), Strongly adherent (SA), ODc (optical density for control), OD (optical density for test)

Table 2. Classification of *S. pyogenes* biofilms

Medium/ Initial inoculum concentration	Control	Day 3	Day 5	Day 7	*P* -value
Parameters	Mean ODc± SD	Mean OD ± SD	Mean OD ± SD	Mean OD ± SD	MHB OD_{600} (0.05) compare with TSB OD_{600} (0.05) **No significance (0.089)**
MHB OD_{600} (0.05)	0.05±0.01	0.76±0.11 **(SA)**	0.53 ± 0.05 **(SA)**	0.05 ± 0.0 **(NA)**	MHB OD_{600} (0.05) compare with TSB OD_{600} (0.1) （**0.013**）
MHB OD_{600} (0.1)	0.05±0.01	1.11±0.01 **(SA)**	0.67 ± 0.04 **(SA)**	0.06 ± 0.01 **(NA)**	MHB OD_{600} (0.1) compare with TSB OD_{600} (0.05) **(0.016)**
TSB OD_{600} (0.05)	0.05±0.01	0.83±0.07 **(SA)**	0.56 ± 0.05 **(SA)**	0.05 ±0.0 **(NA)**	MHB OD_{600} (0.1) compare with TSB OD_{600} (0.1) **No significance (0.110)**
TSB OD_{600} (0.1)	0.05±0.01	1.07±0.02 **(SA)**	0.64 ± 0.09 **(SA)**	0.06 ± 0.01 **(NA)**	TSB OD_{600} (0.1) compare with TSB OD_{600} (0.05) **(0.018)**
					MHB OD_{600} (0.05) compare with MHB OD_{600} (0.1) **(0.014)**

Note: Mueller Hinton Broth (MHB), Tryptic Soy Broth (TSB), Non-adherent (NA), Weakly adherent (WA), Moderately adherent (MA), Strongly adherent (SA), ODc (optical density for control), OD (optical density for test)

crotiter plate after 3, 5, and 7 days of incubation with MHB OD 0.05, MHB OD 0.1, TSB OD 0.05, and TSB OD 0.1. The biofilms adhered strongly to the plate on days 3 and 5 but did not adhere on day 7. As *P. aeruginosa* and *S. pyogenes* biofilms on day 3 have displayed the strongest adherence for both media and densities therefore, we further quantify statistically. The formation of *P. aeruginosa* biofilm in MHB OD 0.1 was significantly higher than MHB OD 0.05 ($p = 0.014$) and TSB OD 0.05 ($p = 0.016$). Meanwhile, the formation of *S. pyogenes* biofilm in MHB OD 0.05 was significantly higher than MHB OD 0.1 ($p = 0.016$) and TBS OD 0.1 ($p = 0.019$).

4 Discussion

This is the first comparative study between MHB OD 0.05, MHB OD 0.1, TSB OD 0.05, and TSB OD_{600} (0.1) for biofilm formation by *P. aeruginosa* and *S. pyogenes*. Biofilm formation in microtiter plates is certainly the most commonly used method. Originally developed by Fletcher to investigate bacteria attachment (Azeredo et al., 2017; Fletcher, 1977), it further proved to be compatible with the study of sessile development (Azeredo et al., 2017). In the classical procedure, bacterial cells are grown in the wells of a microtiter plate (Djordjevic et al., 2002). At different time points, the wells are emptied and washed to remove planktonic cells before staining the biomass attached to the surface of the wells. Biofilm biomass can alternatively be quantified by detachment and subsequent plating (Azeredo et al., 2017). Biofilm producing bacteria are responsible for many recalcitrant infections and are notoriously difficult to eradicate. They exhibit resistance to antibiotics by various methods like restricted penetration of antibiotic into biofilms, decreased growth rate and expression of resistance genes (Lewis, 2001). There are various methods for biofilm detection (Aparna and Yadav, 2008; Donlan et al., 2001; Hassan et al., 2011). In this study, *P. aeruginosa* and *S. pyogenes* were evaluated by a microtiter plate method for their ability to form biofilms using both media (MHB and TSB) and ODs ($OD_{600\ 0.05}$ and $OD_{600\ 0.1}$). *P. aeruginosa* biofilms on day three were significantly higher compared to days four, five and six. Similarly, *S. pyogenes* biofilms on day three were significantly higher compared to days four, five, and six. Thus, suggest that at day three, a large number of bacteria in the plate were switched to biofilm. An optimum number of bacteria adherence occurred in microtiter plate resulting strong biofilm formation on that day (Jama et al., 2017; Rossi et al., 2016). In addition to day three, this study indicates that the biofilm formation by *P. aeruginosa* and *S. pyogenes* were strong on days four, five and six. *P. aeruginosa* and *S. pyogenes* were not able to produce strong biofilm on days two and seven. Many factors such as integration of diverse signals from the environment might play a role in biofilm formation, concurrent with other events such as phenotypic and genetic switching during biofilm production and also EPS production (Bakar et al., 2018; Ismael, 2013). This study found that both bacteria are able to form biofilms by using MHB and TSB, but *S. pyogenes* biofilm was slightly higher in MHB than TSB. Biofilms can form until day five by using both media (MHB and TSB) at both ODs ($OD_{600\ 0.05}$ and $OD_{600\ 0.1}$). Composition of medium has been documented to influence the ability of bacteria to produce biofilm under *in vitro* conditions. The presence of glucose in growth media has been reported to enhance biofilm formation (Stepanovi et al., 2000). Type of media, culture condition and surface of ves-

sel cultivation are significantly robust the biofilm formation (Hood and Zottola, 1997; Iversen et al., 2004). The ability to adhere to a solid surface and the consecutive formation of an organised bacterial biofilm community are crucial for the formation of *P. aeruginosa* and *S. pyogenes* biofilms. This is because the formation of biofilm depends on the ability of bacteria to attach in the surface for 96-well plate (Merritt et al., 2011). It is well known that the switching from a planktonic to a biofilm mode of growth is an intricate process, which occurs in response of environmental changes. As the first step of biofilm formation is bacterial adhesion to surface, this study hypothesis that the strains showed a high ability to create hydrophobic interactions with the microtiter plate surface (Woo et al., 2012). Moreover, physical and chemical plate properties are the main factor that regulate the initial adhesion process (Lemos et al., 2014).

Comparison between Mueller-Hinton Broth (MHB) and Tryptic Soy Broth (TSB) for *Pseudomonas aeruginosa* and *Streptococcus pyogenes* biofilm formatio Recognition of the fact that bacterial biofilm may play a role in the pathogenesis of disease has led to an increased focus on identifying diseases that may be biofilm-related. Biofilm infections are typically chronic in nature, as biofilm-residing bacteria can be resilient to both the immune system, antibiotics, and other treatments (Vestby et al., 2020). In most cases reviewed, the biofilms were identified through various imaging technics, in addition to other study approaches. Biofilm being a mere reservoir of pathogenic bacteria, to playing a more active role, e.g., by contributing to inflammation. Observations also indicate that biofilm does not exclusively occur extracellularly, but may also be formed inside living cells. Furthermore, the presence of biofilm may contribute to development of cancer (Vestby et al., 2020). Commonly, biofilm formation is influenced by cell motility particularly when it is mediated by flagella. Under certain environmental conditions, flagella is necessary for biofilm formation by *P. aeruginosa* (Priya and Brundha, 2013). However, the rapid decrease of biofilm-forming capacity that observed on day seven could be attributed to the loss of exopolymers from the biofilm and in particular of exopolysaccharides, which may suggest that an active process of detachment was occurring, probably mediated by enzymatic degradation (Allison et al., 1998). Biofilms can be found almost anywhere and may impact human health both positively and negatively. One example of a positive effect includes the biofilms of commensal bacteria such as *S. pyogenes* and *S. epidermidis*, which can impede the colonisation of potentially pathogenic bacteria through the stimulation of host-cell immune defences and the prevention of adhesion (Gupta et al., 2016; Vestby et al., 2020). Due to the widespread distribution of biofilms in diseases and their resilience to numerous antimicrobial treatments, biofilm research is receiving more attention. Owing to increasing antimicrobial resistance, the focus of current research is shifting from targeting bacterial growth/division that causes cell death or dormancy, towards novel approaches (Archer et al., 2011).

Previous study showed that *P. aeruginosa* produced a great biofilm on day three (Culotti and Packman, 2014). Another study found that day three was the preferable day in producing strong biofilm by *Proteus mirabilis* (Emineke et al., 2017). A study in 2001 showed that an ideal cultivation period for producing biofilms by *Candida albicans* was at 72 hours and *Saccharomyces cerevisiae* was at 60 hours (Chandra et al., 2001). Previous study reported that *Escherichia coli* produced a strong biofilm on day 3 and 5 (Al-kafaween et al., 2019b). Study by (Jaffar et al., 2016) reported that day 2 was the preferable incubation duration to produce biofilm in *A. actinomycetemcomitans* and day 7 for *P. gingivalis*. Previous studies have indicated that temperature, nutrients and other components in media affected the attachment of microorganisms to the surfaces of various materials (Hood and Zottola, 1997; Iversen et al., 2004). Study by (Al-kafaween et al., 2019b) showed that the *E. coli* produced biofilm on day 3 and day 5 in Brain heart infusion (BHI) and Luria broth (LB) at OD 0.05 and OD 0.1 (Al-kafaween et al., 2019). Studies have used TSB for *Staphylococcus* and *Pseudomonas* species but this depends on growing condition of microorganisms and optical density (Nyenje et al., 2013; Stepanovi et al., 2000). Meanwhile, *P. aeruginosa* and *Klebsiella pneumonia* have been reported to produce strong biofilm formation in TSB in different pH and different temperatures (Hoštacká et al., 2010). Previous study demonstrated that the *S. epidermidis* had produced biofilm on day 3 and 5 in MHB and TSB at OD 0.05 and OD 0.1 (Al-kafaween et al., 2019b). This study suggests that as non-motile bacteria, *S. pyogenes* are able to form strong biofilm with very minimum number of CFU. On the other hand, *P. aeruginosa* is a motile bacterium prefers more cells for biofilm formation. However, both concentration of OD 0.05 and OD 0.1 are ranged as lower concentration of CFU. Lower concentration is the best start for biofilm maturation because it provides enough attachment space for planktonic cells (Jaffar et al., 2016). However, in this study selected the suitable media based on the highest value of biofilm formation after the measurement of wavelength was obtained regardless the level of inoculum. However, based on our findings, MHB is most suitable for biofilm formation by *P. aeruginosa* and *S. pyogenes.* The type of media, culture condition, and surface of vessel cultivation are significant factors affecting robust biofilm formation. Composition of the medium can also influence the ability of bacteria to produce biofilm under *in vitro* conditions.

5 Conclusion

Incubation time, growth medium, and OD can significantly affect the formation of *P. aeruginosa* and *S. pyogenes* biofilms. We found that the formation of biofilm by *P. aeruginosa* and *S. pyogenes* successfully occurred on day 3. MHB is a suitable medium for *P. aeruginosa* and *S. pyogenes* biofilms. Both of these bacteria successfully formed biofilms at lower cell

density such that for *P. aeruginosa* and *S. pyogenes* were 0.1 and 0.05, respectively. We suggest that the specific medium (MHB) and lower cell concentration should be used for formation of strong biofilm by these bacteria and may further facilitate future biofilm research.

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Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data.

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