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Characterization of biofilm formation by *Escherichia coli*: An *in vitro* study

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ABSTRACT

Objective: The purpose of the present study was to characterize the effects of medium, optical density (OD), and incubation time on biofilm formation by *Escherichia coli* in brain heart infusion (BHI) and Luria-Bertani broth (LB). **Methods:** The main procedure involved 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 OD of the solution at 570 nm 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 0.55–0.35 and 0.70–0.39 in BHI and LB, respectively, at OD 0.05. Similarly, pattern in results was noted for OD 0.1 in both media BHI and LB. **Conclusion:** It is confirmed that 3 days (72 h) are required for obtaining effective biofilm formation in both BHI and LB at 37°C at OD 0.05 and 0.1.

1. INTRODUCTION

Biofilm formation as a bacterial defense mechanism is difficult to break-in with traditional medical treatment tools such as antibiotics. The integrated colonization of bacteria on the surface of the host which is supported by the thick extracellular secretions termed as a biofilm [1,2,3]. Biofilms are more resistant toward antibiotics compared to its planktonic state [1,4] and account for >80% of infections in human body [3]. Hence, it is of crucial importance to understand biofilm formation and associated factors that can effectively be controlled in designing new clinical treatments.

Biofilm formation of pathogenic strains of *Escherichia coli* is considered to be a virulence factor in a host with compromised immune system [5,6,7]. Biofilm formation is well studied in *E. coli*; however, a greater genomic variability coupled with diverse environmental niches leaves a huge task for biofilm formation mechanism exploration [5,7]. Based on its genetic variation, this species has been divided into six phylogenetic groups, i.e., A, B1, B2, C, D, and E using triplex PCR phylogroup assignment methods [6,7,8]. Hence, it is quite diverse in its mode of habitat and defense mechanisms. It is critically important to model *in vitro* study to reveal its biofilm formation patterns.

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In this study, an *in vitro* model study has been designed to investigate the effect of media type, initial optical density (OD), and incubation time in two different growth media, i.e., brain heart infusion (BHI) and Luria-Bertani broth (LB) for better understanding of biofilm formation by *E. coli* [9]. BHI medium is useful for cultivating a wide variety of microorganisms since it is a highly nutritive medium [9]. BHI is also used for the preparation of inoculum in antimicrobial susceptibility testing. While Luria-Bertani broth (LB) is generally used for molecular and genetic studies due to its nutritive capacity and simple composition that can be easily modified. Moreover, LB is used for the cultivation and maintenance of recombinant strains of *E. coli*, originally derived from *E. coli* strain K12, deficient in Vitamin B production. LB is a nutritionally rich medium due to the presence of casein enzymic hydrolysate and yeast extract [10]. This allows their recombinant strains of *E. coli* to grow more rapidly since all the essential growth nutrients required by these strains are readily available; thus, there is no need to synthesize it [11]. Therefore, in this study, the biofilm formation has been compared in two media, i.e., BHI and LB at varying OD with the following hypothesis;

H_1: Initial turbidity of the bacteria (OD) has no significant effect on biofilm formation.
H_2: Incubation time (days) has no significant effect on biofilm formation.
H_3: Interaction of initial turbidity of the bacteria (OD) and incubation time (days) has no significant effect on biofilm formation.

2. MATERIALS AND METHODS

2.1. Bacterial Growth

E. coli strain (ATCC 25922) commonly associated with catheter-associated infections and food safety danger was used in this study. *E. coli* was grown in BHI culture media. After inoculation, broth was incubated at 37°C for 24 h. From these cultures, a suspension of *E. coli* (0.1%) was prepared in BHI broth and 100 µL of this suspension was pipetted into the wells of the 96-well plate [12,13]. These plates were incubated for 24, 48, and 72 h at 37°C. The supernatant from wells, containing planktonic bacteria, was gently aspirated to clear flat-bottomed 96-well plates for the measurement of absorbance at 570 nm.

2.2. Biofilm Assay

For biofilm assay, bacterial growth media and cells were removed and wells were rinsed 3 times with 150 µL of sterile distilled water without disturbing the adherent biofilm. The plates were air-dried for 5 min. Wells were attained with 120 µL of crystal violet (0.1%) for 15 min at room temperature [13]. The crystal violet was removed and the wells were rinsed 3 times with 150 µL of distilled water and left to air dry. Subsequently, 150 µL of 95% ethanol per well was applied and the plates were incubated at room temperature for 15 min. The contents of each well were thoroughly mixed and 125 µL of the crystal violet/ethanol solution was transferred to a clear flat-bottomed 96-well plate. The extent of biofilm formation was determined by measuring absorbance at 570 nm [2,12,14,15].

2.3. Biofilm Analysis

The cutoff was defined as three standard deviations above the mean OD of the negative control (ODc) which contained broth only. The following criteria were used to classify the different adherent strength: $OD \leq OD_C$ = non-adherent, $OD_C < OD \leq (2 \times OD_C)$ = weakly adherent, $(2 \times OD_C) < OD \leq (4 \times OD_C)$ = moderately adherent, and $(4 \times OD_C) < OD$ = strongly adherent [16].

2.4. Statistical Analysis

All the trials were conducted in triplicate to calculate the mean and standard deviations of the data collected. One-way repeated measure ANOVA was conducted to compare the effect of incubation time on biofilm formation in BHI and LB at 0.05 and 0.1 initial OD values [13,17].

3. RESULTS AND DISCUSSION

Biofilm formation was noted to be higher in the first 3 days of incubation in both media (BHI and LB), then it started to slow down till on the 7th day; biofilm formation was negligible [Figure 1]. Further, it was noticed that OD 0.05 and 0.1, in both BHI and LB, 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 LB is more effective at OD 0.05 than HBI, whereas HBI was more effective at 0.1 OD in biofilm formation progress.

3.1. Biofilm Characterization

Based on 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 3 days of incubation at both OD 0.05 and 0.10 for both media (BHI and LB).

To further explore the relationship, if any, between OD values and media, two-way ANOVA was performed, wherein variable factors were OD values and incubation time (days) [Table 2]. It was found that OD has a significant effect on biofilm formation ($P = 0.0106$ and $P < 0.05$). Therefore, null hypothesis H_1 is rejected. Similarly, days of incubation had significant effect on biofilm formation ($P < 0.05$); reject the second hypothesis. However, it was observed that the interaction of OD values and days of incubation had a non-significant effect on biofilm formation (H_3).

To further investigate which day and OD concentration is more effective in biofilm formation, Tukey's multiple comparison test was also performed to check the significance of time (days) on biofilm formation in both media. It showed that the time (days) had a significant effect ($P < 0.05$) on biofilm formation [Table 3] under both 0.05 and 0.10 OD [13].

Therefore, it can be concluded that 72 h of incubation at 37°C is the ideal time for biofilm formation in BHI and LB for *E. coli*. Similar study was conducted by Low *et al*., [9] where the growth of *E. coli* was monitored for 11 weeks in three different media.

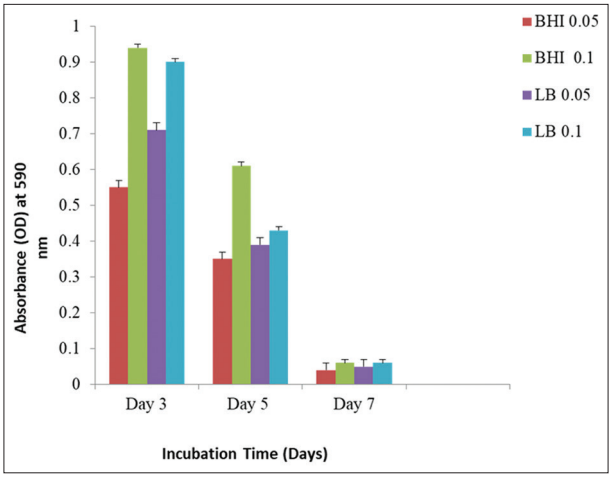

Figure 1: Biofilm formation by *Escherichia coli* in brain heart infusion and Luria-Bertani broth media

Table 1: Characterization of biofilms based on OD values

Times (day)	Control	BHI 0.05	BHI 0.1	LB 0.05	LB 0.1
Day 3	0.06	0.55 SA	0.94 SA	0.71 SA	0.9 SA
Day 5	0.06	0.35 SA	0.61 SA	0.39 SA	0.43 SA
Day 7	0.06	0.04 WA	0.06 WA	0.05 WA	0.06 WA

$OD \leq OD_C$=Non-adherent, $OD_C < OD \leq (2 \times OD_C)$ = Weakly adherent, $(2 \times OD_C) < OD \leq (4 \times OD_C)$ = Moderately adherent, and $(4 \times OD_C) < OD$=Strongly adherent. BHI: Brain heart infusion, LB: Luria-Bertani broth

Table 2: Two-way ANOVA for determining critical factors in biofilm formation

Factors	SS	DF	MS	F (DFn, DFd)	*P* value
Interaction	0.03763	2	0.01882	F (2,6)=3.715	*P*=0.0892
OD	0.06785	1	0.06785	F (1,6)=13.40	*P*=0.0106*
Incubation days	1.05	2	0.5251	F (2,6)=103.7	*P*<0.0001*
Residual	0.03039	6	0.005065		

SS: Sum of squares, DF: Degree of freedom, MS: Mean square F (DFn, DFd) = F distribution, *P<0.05 considered statistically significant

Table 3: Two-way ANOVA with Tukey's multiple comparison tests

Tukey's multiple comparison	Mean 1	Mean 2	Mean diff.	SE of diff.	Adjusted *P* values
Day 3 versus day 5	0.7736	0.445	0.3286	0.097	0.1372
Day 3 versus day 7	0.7736	0.05	0.7236	0.097	*0.0318
Day 5 versus day 7	0.445	0.05	0.395	0.097	0.0992

*Significant

4. CONCLUSION

Time interval for biofilm formation is critical in BHI and LB. The biofilm formation growth follows a sinusoidal pattern, wherein a peak of biofilm formation achieved in 72 h of incubation and then decline gradually until the 7th day. Overall, it can be deducted from this study that BHI and LB can give maximum biofilm formation by *E. coli* within 72 h of incubation period. However, further, investigation is recommended to reveal the biofilm formation pattern in *E. coli* under variable environment to model better remedial pathways in controlling infections. Moreover, a quantitative analysis of biofilm can reveal structural component of biofilm.

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It is declared here by the authors of this article that there are no conflicts of interest in writing and publishing these research findings.

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