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Tolerance of biofilm forming bacteria to thermal shock and detergent

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Abstract
Biofilm forming bacteria play a vital role in ecological balance. The objective of this study was to determine the effect of thermal shock and detergents on the survival of biofilm forming bacteria. The *Escherichia coli* biofilm were subjected to thermal shocks in the range of 60°C to 100°C for duration between 5 to 60 min. Effect of different concentrations (1-10%) of Sodium Dodecyl Sulfate (SDS) on survival of bacteria was also studied. Colony forming units (CFU) were quantified using direct enumeration method. Reduction of CFU concentration with increasing temperature was observed. *E. coli* cells were able to survive at 60°C up to 20 min duration, while at 70°C the bacteria could not survive even for 5 min. Increasing SDS concentration also resulted in reduction of bacterial colonies. Biofilm forming *E. coli* demonstrated tolerance to high thermal shock and detergent. This can assist in stabilization of anthropogenic-induced environmental imbalance.

Keywords: Anthropogenic factors, bioremediation, CFU, Escherichia coli, heat resistance

1. Introduction
Biofilms are complex community of microorganisms [1] enclosed by a matrix of extracellular polymeric substance (EPS). The microbial cells growing in such communities may adhere on a wide variety of surfaces, including natural aquatic systems, medical implants, water pipes, dental plaques or living tissues such as those of animals and plants. A mature biofilm has a defined architecture, consists of microbial cells of the same or different species and EPS. This organization provides a favorable environment for exchange of genetic material, communication signals and metabolites between the microbial cells [2]. Biofilm plays an important role in nitrification [3] and cycling of sulfur and metals. The environmental and industrial application of these biofilms are reflected in degradation of organic matter and environmental pollutants, sewage processing, water purification in water-treatment plants, treatment of petroleum contaminated groundwater and detoxification of contaminated areas of the environment [1]. However, biofilms are also known for their association with a variety of diseases and device-related infections. Biofilm-grown cells are physiologically different from planktonic cells and express distinct properties, one of which is an increased resistance to antimicrobial agents [4].
Anthropogenic factors constitute the changes introduced into nature by human activities. The primary anthropogenic factors produce ecological and genetic effects contributing to extinction risk and disturbing natural balance of an ecosystem [5]. Moreover, human activities are responsible for introduction of a wide range of pollutants into natural water bodies via industrial, household and agricultural activities. The aquatic life is also affected by alteration in water temperature, water flow, nutrient concentration, addition of detergents including several other factors. Biofilms have been found to be suitable for remediation strategy of pollutants because of their high microbial biomass and ability to immobilize pollutants [6].
This study was based on the hypothesis that the attachment of bacteria to a surface and subsequent formation of biofilm, exemplifies a survival strategy during anthropogenic-induced environmental imbalance. Few studies have been conducted till date related to this hypothesis. The objective of this study was to determine the effect of thermal shock and detergents on the survival of biofilm forming bacteria. *Escherichia coli* were used as the model organism and Sodium Dodecyl Sulfate (SDS) as the model detergent.

2. Materials and methods
2.1 Bacteria and media
The biofilm forming *Escherichia coli* (ATCC 10536) was used in this study. The bacteria were maintained on Tryptone soya agar (TSA) slants with periodical subculturing and stored at 4°C. The purity was examined at regular intervals by gram staining and biochemical test.

2.2 Biofilm formation assay
For biofilm assays, the bacteria biofilms were cultured in Petri plates containing two glass microscopic slides. The bacterial cultures were standardized to an average cell density of 1 x 10^6 CFU/mL. Tryptone soya broth (TSB) was added to the Petri plates along with inoculum in a 5:0.33 mL ratio. The Petri plates were placed in an incubator for 48 h at 37°C for the growth of bacteria and development of biofilm. After incubation, qualitative assessment of biofilm formation was determined by crystal violet binding assay using 0.1% (v/v) crystal violet solution at room temperature [7].

2.3 Evaluation of thermal shock
After incubation, TSB was removed using a glass pipette and the slides were rinsed in 0.1% (v/v) peptone water. Fresh TSB was added carefully to each Petri plates and slides were subjected to thermal shock. One slide from each petriplate was reserved for heat shocking at control conditions. The heat shock temperatures investigated were 37°C (for control evaluation purposes), 60°C, 70°C and 80°C and the heat shock durations were 5, 20, 40 and 60 min [8]. Immediately following the thermal shock, the biofilms were mechanically removed

with a spatula [9] and resuspended in sterile distilled water to homogenize the biofilm.

2.4 Evaluation of detergent tolerance

The anionic surfactant sodium dodecyl sulfate (SDS) was used for this study. After the development of biofilm, the slides were removed from Petri plates and immersed in another Petri plates containing SDS at concentrations of 1%, 5% and 10% and gently stirred for 30 min at 37°C. The slides were rinsed in 0.1% (v/v) peptone water. The biofilms were mechanically removed with a spatula immediately after the treatment and resuspended in sterile distilled water. The entire experiment was carried out in three repetitions and the analyses in triplicate.

2.5 Quantification via direct enumeration

A 100 µl of homogenized bacterial suspension was taken and serial dilutions were prepared using sterile distilled water. Plating was done on TSA plates using the spread plate technique to determine the number of viable cells. Subsequently, the Petri dishes were incubated at 37°C for 24 h and then the plate standard count was carried out for each plated dilution and expressed in the CFU/ml. From the collected plate counts, the number of viable bacteria in the biofilm was back-calculated based on CFU count per plate and the dilution factor [10, 8]. All analyses are performed on the log (CFU) using the following equation.

$$\log\left(\frac{CFU}{cm^2}\right) = log\left(\left(\frac{plate\ count * 10^{dilution\ factor}}{0.1\ ml}\right) X \left(\frac{15\ ml}{slide}\right) X \left(\frac{slide}{18.75\ cm^2}\right)\right)$$

3. Results & Discussion

3.1 Biofilm formation assay

E. coli cells were incubated in TSB at 37°C for 48 h produced biofilm on the glass slides as visualized by crystal violet binding assay. Biofilm formation has been reported in both commensal and pathogenic *E. coli* by Beloin *et al.* [11]. The *in vitro* biofilm formation of commensal and pathogenic *E. coli* strains varies widely and is dependent on the applied growth condition. However, good biofilm formation was not found to be associated with disease-associated isolates [12].

3.2 Evaluation of thermal shock

After the formation of biofilm, the *E. coli* cells were tested for their ability to withstand thermal shock at different temperature ranging from 37°C (for control evaluation) and by elevating the temperature to 60°C, 70°C and 80°C. At each temperature, durations for 5, 20, 40 and 60 min were evaluated (Table 1). The overall trends of log CFU reduction and the sensitivity to thermal shock time and temperature combinations were quantified via direct enumeration. It was observed that *E. coli* cells were able to withstand a temperature of 60°C up to 20 min duration. However, increasing temperature to 70°C had affected the stability of cells, as indicated by zero CFU count (Table 1). Moreover, thermal shock at 80°C resulted in complete deactivation of the biofilms and the cells could not survive. The data was reported as the average log (CFU/cm^2) ± standard deviation. Overall, it was found that, as thermal shock temperature increased, the average log (CFU/cm^2) decreased (Table 1).

Table 1: Average log (CFU/cm^2) ± standard deviation of biofilms as a function of thermal shock temperature and duration.

Dilution	Temperature	Duration			
		5 min	20 min	40 min	60 min
10^{-4}	37°C	6.89 ± 0.06	7.26 ± 0.04	7.43 ± 0.02	7.45 ± 0.03
	60°C	6.62 ± 0.04	5.16 ± 1.13	NC	NC
	70°C	NC	NC	NC	NC
10^{-6}	37°C	7.55 ± 0.15	7.78 ± 0.09	7.85 ± 0.09	8.05 ± 0.06
	60°C	7.26 ± 0.31	NC	NC	NC
	70°C	NC	NC	NC	NC

*NC = No Colony forming units

The dynamics of *E. coli* near the maximum temperature for growth in a rich medium was analyzed previously [13]. 'Smooth' growth curves were observed at 40°C to 43°C, based on viable counts, while at 44°C to 46°C the exponential growth phase were interrupted and showed a non-exponential growth which may seem related to protein destabilization. The growth of *E. coli* beyond 40°C is generally prohibited. But it can grow at elevated temperatures of 45°C for 5 min and can survive consistently at a temperature as high as 49°C, may be by acquiring mutations. Moreover, *E. coli* growth was also reported up to 53°C but it was sporadic and not reproducible [14]. Investigators earlier reported high water temperature as an important factor in reducing the survival of *E. coli* bacteria in the water phase in drinking water pipes [15, 16].

In this study we observed that the *E. coli* survived at 60°C, possibly because of the presence of biofilm, since the thermal shock was subjected after biofilm formation of 48 h cultivation. Our study suggested possible role of biofilm in providing stability of *E. coli* cells to elevated temperature as high as 60°C.

3.3 Evaluation of detergent tolerance

The biofilm behavior after treatment with different concentrations of SDS (1%, 5% and 10%) was studied in terms of log CFU count. It was observed that *E. coli* cells were able to survive at 10% SDS concentration, indicating that the biofilm formed on glass slides were unaffected by the interfacial tension of SDS and thus showed surfactant-resistance property. However, the results revealed reduction in average log (CFU/cm^2) with increasing SDS concentration (Table 2). Increase in the SDS concentration possibly aided in the physical removal of *E. coli* cells by lowering the surface tension, thus resulting in reduction of log CFU count as shown in Table 2. High concentration of surfactant could reduce the

adhesive property of bacterial strains and thus biofilm formation [17]. The data of this study indicated that the biofilm produced by the *E. coli* strain under study had higher surface interaction. This can be used as bio-surfactant in environmental bioremediation [18].

Table 2: Average log (CFU/cm^2) ± standard deviation of biofilms as a function of SDS concentration

Dilution	SDS concentration	Average log (CFU/cm^2) ± S.D.
10^{-4}	1%	6.60 ± 0.06
	5%	6.06 ± 0.07
	10%	5.76 ± 0.08
10^{-6}	1% -10%	NC

*NC = No Colony forming units

The development of biofilm communities with similar species or with other microbes extend the ability of microbes to adapt and flourish in even the most hostile environments as well as from environmental alterations via natural or anthropogenic factors. Biofilms have been implicated as an important reservoir for pathogens and commensal. A study conducted by Culotti and Packman [19] demonstrated that *E. coli* not only persists in aquatic biofilms under depleted nutritional conditions, but interactions with *Pseudomonas aeruginosa* can greatly increase *E. coli* growth in biofilms under experimental conditions. In multispecies biofilms, interspecies interactions may greatly alter the response of the community to various toxic exposures such as protection against the action of disinfectants [20]. Such protective function may be associated with specific components produced by one species that benefit the whole population [21]. For instance, secretion of a specific hydrolase enzyme by *P. aeruginosa* in the matrix was found to confer tolerance to SDS on a mixed community [22]. Thus multispecies biofilm can be used as a tool for bioremediation of industrial and domestic effluents. Since the strain under study can withstand high thermal shock and high surfactant it can be applied with multiple potent species for bioremediation process.

4. Conclusions

This study demonstrated tolerance of biofilm forming bacteria towards heat shock and detergent. The CFU concentration of *E. coli* cells after stress condition revealed that the strain can withstand high thermal shock even at 60°C. Additionally, the strain tolerated the interfacial tension even at 10% SDS, as indicated by the viability of bacteria in this biofilm. This potency of biofilm can be widely applied in different bioremediation activities. Further studies are needed to evaluate the stability of this biofilm with multiple species which have different mechanism under thermal and surfactant exposure. Study on the efficacy of multispecies in biofilm would facilitate the development of a better bioremediation of polluted sites and wastewaters.

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