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Research Article

Differential biofilm formation and chemical disinfection resistance of *Escherichia coli* on stainless steel and polystyrene tissue culture plate

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ABSTRACT

Background: Biofilms are aggregates of microbial cells enclosed in an extracellular polymeric substance and attached to surfaces. Biofilm formation and its resistance to antimicrobials is becoming a serious challenge in food industries and hospital settings. The aim of this work was to study the formation of biofilm by *E. coli* on Stainless steel (SS) and Polystyrene Tissue Culture Plate (TCP) at 10 and 27°C, and also to assess the action Hydrogen Peroxide (HP), Para Acetic Acid (PAA), Sodium Hypochlorite (SH) and mixture of PAA + SH disinfectants against the biofilm.
Methods: 200 µL of 10^8 suspension of *E. coli* ATCC 29922 was inoculated on the SS and into the wells of TCP, incubated at 10 and 27°C for 24, 48 72 and 168 hours. Biofilm developed at each incubation hour above was quantified by bead-vortex method followed by agar plating. The action of disinfectants was tested on 168 hours biofilm. The surfaces were exposed to the disinfectants and incubated at 27°C for 10 minutes, followed by deactivation for 5 minutes. Cells that resisted disinfectants action were vortexed and enumerated by agar plating.
Results: From the results *E. coli* developed higher biofilm on SS than TCP at 72 hours and 27°C. After disinfection, HP was the most effective with log reduction value of 1.11 followed by PAA (1.07), then PAA + SH (1.04) while SH was the least (0.92).
Conclusions: The result of this work showed that HP and PAA can be good disinfectants against *E. coli* biofilm.

Keywords: Biofilm, *E. coli*, Log reduction, Disinfectants

INTRODUCTION

Biofilms can be defined as microbial cells immobilized in a matrix of extracellular polymers acting as an independent functioning ecosystem, homeostatically regulated".[1] The Extracellular Polymeric Substance (EPS) act as a barrier that shields the enclosed cells from the bactericidal action of many antimicrobial agents and phagocytic action of host immune defenses leading to increase resistance to antimicrobials and/ or disinfectants and persistent infections.[2,3] Thus these microorganisms are of great interest to the area of health and especially those that have been adapted to harsh environments, developing mechanisms against the bactericidal agents.[4,5] Biofilms are notoriously difficult to remove and are related to 80% of all human infections.[6] The ability of

microorganism to adhere to surfaces and to engage in a multistep process leading to biofilm formation is also a serious problem to food industries leading to food spoilage and cross contamination as well as outbreaks of foodborne diseases.

Microorganism in biofilms are resistant to various types of stress, including disinfection.[7] The increased resistance of biofilm cells to biocides, which is at least partially because of interference of the exopolymeric explains why the disinfectant most effective to planktonic cells is not necessarily the most active against biofilm cells.[8] It has since been characterized in several laboratories as causing self-limiting diarrhea, hemorrhagic colitis, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura in children and other susceptible groups of individuals.[9,10] Outbreaks of *Escherichia coli* (*E. coli)* O157:H7 infections have been primarily associated with eating undercooked ground beef, but a variety of other foods have also been implicated as vehicles.[11] Cross-contamination of foods can occur in food-processing plants and during subsequent handling and preparation, resulting in a wide range of foods being implicated in outbreaks of *E. coli* O157:H7 infections.[12,13]

Escherichia coli (*E. coli*) had been an important Gram negative model for in vitro analysis of biofilm formation on abiotic surfaces.[14,15] *E. coli* are genetically diverse species that causes diarrheal diseases and variety of extraintestinal infections which fulfill many or all of the proposed criteria for biofilm-associated infections.[16] The organism has emerged with increasing frequency as a food borne pathogen of concern over the last 20 years and is responsible for causing serious illness and sequelae in susceptible humans.

When bacteria are exposed to sub-lethal levels of antimicrobials or biocides, only minor cell damage is caused and the consequences of that may include changes in their phenotype and induction of gene expression, giving rise to a more resistant population.[18] Thus the aim of this work was to study the development of biofilm formation by *E. coli* on SS, TCP at 10 and 27°C, and also to evaluate the efficacy of four different chemical disinfectants namely Hydrogen Peroxide (HP), Para Acetic Acid (PAA), Sodium Hypochlorite (SH) and mixture of PAA with SH against *E. coli* biofilm.

METHODS

Bacterial strain and culture condition

E. coli strain ATCC 29922 was used for the study and was grown on Tryptic Soy Agar (TSA) overnight at 37°C and stored at 5°C for further experiment.

Preparation of test surfaces

The stainless coupons (3 cm x 1 cm) were degrease in acetone for 1 hour to remove any manufacturing debris, washed in detergent solution, rinsed twice with distilled water, air-dried and autoclaved at 121°C for 15 minutes prior to use.[19] The TCP (SPL life sciences Korea) were aseptically kept clean for use in the experiment.

Preparation of inoculum

One colony from the overnight cultures was picked and inoculated into 5 ml of Tryptic Soy Broth (TSB) in a 50 ml tube and incubated at 37°C for 2 hours. After 2 hours of incubation, 2 ml of the incubated strain were inoculated into 200 ml of TSB in a conical flask, incubated in an orbital shaker at 37°C for 16 hours.[19] After 16 hours of incubation, 10 ml of the incubated strain were centrifuged at 5000 x *g* for 5 min at 10°C, washed twice in 10 ml of Phosphate Buffer Saline (PBS) (pH 7.3).[20] The cell pellets were re-suspended in 10 ml of TSB to an optical density of 0.5 at 600 nm (OD_{600}) which corresponds to approximately 10^8 CFU/ml.[21]

Biofilm formation on stainless steel

Biofilm formation on SS coupons was conducted using the method described by Kostaki et al.,[20] with some modifications. 200 µl aliquot of 10^8 CFU/ml suspension of *E. coli* strain was inoculated on each of the coupons inside petri dishes and allowed to attach for 3 hours at room temperature. Following the attachment step, 20 ml of TSB was introduced into each of the petri dishes containing the coupons. The surfaces were incubated at 10 and 27°C for 24, 48, 72 and 168 hours periods to allow for biofilm development.

Biofilm formation on tissue culture plate (TCP)

Biofilm formation on TCP was carried out by pipetting 200 µl aliquot of 10^8 CFU/ml suspension of the *E. coli* into 9 wells of sterile 96-well TC plates while control wells were only filled with broth. The plates were later incubated at 10, 27 and 37°C for 24, 48, 72 and 168 hours in order for the bacteria to attach to the pirates of the wells. Following the attachment step, the planktonic bacteria were removed using a multichannel pipette and each well was washed twice with 200 ml of PBS (pH 7.3) to remove the loosely attached cells. After the removal of loosely attached cells, the wells were renewed with 200 µl of TSB every 24 h up to the 7^{th} day (168 hours) to allow for bacterial growth and biofilm development.

Enumeration of biofilm cells

The enumeration of viable biofilm cells on SS and TCP was conducted after 24, 48, 72 and 48 hours using bead vortexing method described by Giaouris and Nychas.[22] Initially, the coupons were carefully removed from the petri dishes using sterile forceps, rinsed twice by pipetting with 10 ml of PBS, with shaking in order to remove the loosely attached cells. After the second rinsing step, each coupon was individually transferred into 50 ml plastic tube containing 10 ml physiological

saline (0.95% NaCl w/v). The plastic tube was vortexed for 2 min at maximum speed to detach biofilm cells from the coupons. Detached cells were subsequently enumerated by agar plating. Ten-fold of six serial dilutions were made and 100 µl from the sixth dilution was pipetted onto TSA and incubated at 37°C for 24 hours. Finally plates were removed after 24 hours of incubation. Developed colonies were counted taking a range of 3-300 while viable cells were expressed colony forming unit per mill (CFU/ml). The experiments were repeated three times.

Activities of disinfectants against 168 hours biofilm developed on stainless steel

The disinfectants used in this study include Hydrogen Peroxide (HP) 30% (R and M, Essex, U.K), Para Acetic Acid (PAA) 10% (R and M, Essex, U.K), Sodium Hypochlorite (SH) 10% (R and M, Essex, U.K) and mixture of PAA and SH. After 168 hours biofilm development, the coupons were rinsed twice with 10 ml of Phosphate Buffer Saline (PBS) pH (7.3) to remove any loosely attached bacterial cells, placed in new Petri dishes containing 20 ml of each of the disinfectants under study at 27°C for 10 minutes with gentle shaking.[23] A positive control was treated by placing a coupon in a Petri dish containing 20 ml of sterile physiological saline. After 10 minutes, the actions of the disinfectants was deactivated by transferring the coupons into separate petri dishes containing 10 ml of TSB and were allow to stand for 5 minutes.[24] After deactivation, the coupons were rinsed twice again with 10 ml PBS, placed in plastic tubes containing 10 ml of sterile physiological saline and 2 sterile beads, vortexed for 2 minutes[22] in order to dislodge viable bacteria adhering to the coupons into the physiological saline. The control coupons were treated equally as the test control but with Physiological saline and PBS. To count viable cells, bacteria were re-suspended in 6 dilutions with sterilized physiological saline and cultured on TSA at 37°C for 24 hours.

Activities of disinfectants against 168 hours biofilm developed on polystyrene tissue culture plates

The activities of the disinfectants on TC plates were carried out using the method described by Pitts et al.,[25] with some modification. 200 µl of the disinfectants were pipetted into the different wells designated for each disinfectant. Positive control wells were filled with only TSB. The disinfectants were allowed to act for 10 minutes followed by deactivation for 5 minutes by pipetting 200 µl of TSB into the wells.[24] Positive control wells were equally treated the same way but with only physiological saline.

After 5 minutes of deactivation, the TSB was removed and the wells were re-solubilized with 200 µl of 95% ethanol, allowed to stand for 20 minutes in order to dislodge the biofilm cells adhered to the pirates of the wells. After 20 minutes, the solubilized biofilm cells were pipetted and transferred into 10 ml of normal physiological saline. Survived cells were enumerated by agar plating on TSA after six-fold dilutions were made. Developed colonies were counted and converted into colony forming unit/ml (CFU/ml). The efficacy of the various disinfectants was evaluated by the ratio of untreated to the ratio of treated viable cell x 100 and that gives the percentage survival fractions (%SF) while the Percentage Killed (PK) was evaluated using the formula: PK = (1-SF) x 100% hence the log reduction (LR) factor was evaluated using the formula: LR= Log_{10} (1/SF).[26]

RESULTS

Biofilm formation at 10°C

Assessment of biofilm developed by *E. coli* on different surfaces at different incubation temperatures and time showed varied results. High biofilm development was observed on Stainless steel (SS) than Tissue Culture Plate (TCP) after 72 hours of incubation shown in Figure 1.

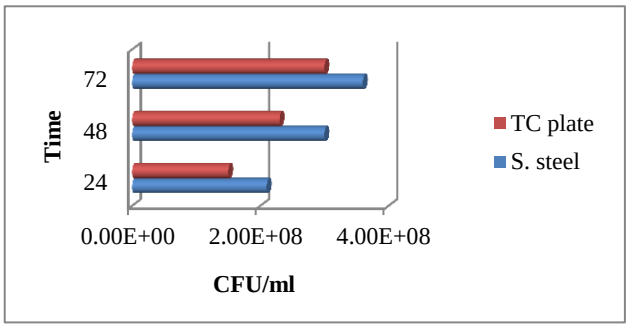

Figure 1: Quantification of *E. coli* viable cells at 24, 48 and 72 hours on stainless steel (SS) and tissue culture plate (TCP) at 10°C.

Biofilm formation at 27°C

Biofilm formation assay by *E. coli* on the two surfaces also showed varied result at 27°C with much increase in biofilm development on the surfaces than the earlier incubation temperature (10°C).

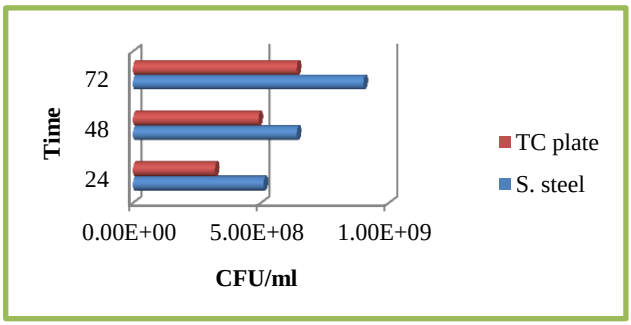

Figure 2: Quantification of *E. coli* viable cells at 24, 48 and 72 hours on stainless steel (SS) and tissue culture plate (TCP) at 27°C.

The increase in incubation hours resulted in higher biofilm formation by *E. coli* with highest biofilm developed at 72 hours. At this incubation temperature, biofilm development was higher on SS than TCP as illustrated in Figure 2.

Efficacy of disinfectants at 10°C

The action of the different disinfectants on the different surfaces has reduced the biofilm compared to the positive control. All the disinfectants had successfully killed the biofilm developed on TCP, while on SS none of the disinfectant was able to kill all the biofilm, although higher reduction in the number of viable cells that rested the bactericidal effect was obtained (Table 1).

Efficacy of disinfectants at 27°C

The bactericidal action of HP, PAA, and PAA + SH at 27°C completely killed the biofilm developed on TCP, with the exception of SH. Biofilm developed by *E. coli* on SS were not completely killed by all the tested disinfectants at this temperature, but high bactericidal action was achieved (Table 2).

Table 1: Efficacy of disinfectants against 168 hours biofilm developed at 10°C.

Disinfectants	Surfaces	Positive control (CFU/ml)	Number of surviving cells (CFU/ml)	LR	% SF	% Killed
Hydrogen peroxide (HP)	S. steel	8.60×10^8	1.00×10^7	1.94	1.16	98. 84
	TC plate	5.80×10^8	-	-	0.00	100
Sodium hypochlorite (SH)	S. steel	8.60×10^8	3.00×10^7	1.03	9.30	90.97
	TC plate	5.80×10^8	-	-	0.00	100
Para acetic acid (PAA)	S. steel	8.60×10^8	1.00×10^8	0.93	11.63	88.37
	TC plate	5.80×10^8	-	-	0.00	100
PAA + SH	S. steel	8.60×10^8	1.10×10^8	0.89	12.79	87.21
	TC plate	5.80×10^8	-	-	0.00	100

Table 2: Efficacy of disinfectants against 168 hours biofilm developed at 27°C.

Disinfectants	Surfaces	Positive control (CFU/ml)	Number of surviving cells (CFU/ml)	LR	% SF	% Killed
Hydrogen peroxide (HP)	S. steel	1.42×10^9	1.10×10^7	1.11	7.75	92.25
	TC plate	1.02×10^9	-	-	0.00	100
Sodium hypochlorite (SH)	S. steel	1.42×10^9	1.70×10^8	0.92	11.97	88.03
	TC plate	1.02×10^9	3.00×10^7	1.53	2.94	97.06
Para acetic acid (PAA)	S. steel	1.42×10^9	1.20×10^8	1.07	8.45	91.55
	TC plate	1.02×10^9	-	1.71	1.96	98.04
PAA + SH	S. steel	1.42×10^9	1.30×10^8	1.04	9.15	90.85
	TC plate	1.02×10^9	-	-	0.00	100

DISCUSSION

Biofilm formation at 10°C

At 10°C after 24 hours of incubation, enumeration biofilm developed by *E. coli* on SS presented a count of 2.10×10^8 CFU/ml, while a less dense biofilm was quantified on TC plates with a count of 1.50×10^8 CFU/ml (Figure 1). Scanning electron micrographs have shown that food-borne pathogens and spoilage microorganisms accumulate as biofilms on stainless steel, aluminium, glass rubber, Teflon seals, and nylon materials typically found in food-processing environment.[27,28]Although polystyrene is not a material used in medical devices, other than for *in vitro* diagnostics, it has been shown to be an excellent material for promoting adherence of cells for tissue culture techniques and bacteria for assessment of biofilm formation.[29,30]

At 48 hours, quantification of biofilm viable cells developed by *E. coli* on SS yielded a count of 3.00×10^8 CFU/ml. The biofilm density quantified on TC plates was also less with a count of 2.30×10^8 CFU/ml. The number of bacterial cells adhered to the stainless steel coupons after 72 hours quantification was much higher than the previous incubation hours presented a count of 3.60×10^8 CFU/ml whilst a count of 3.00×10^8 CFU/ml was

obtained on TCP. Although a relatively high number of viable cells were quantified on TCP, Vidal et al.,[31] and Prigent-Combaret et al.,[32] reported that the nonpathogenic *E. coli* tend to attach better on polystyrene. Bacterial adhesion capacity occurs as a function of the initial inoculum (time 0) and it is a parameter that evaluates the ability of free cells, originating from a liquid medium, to adhere to solid surfaces, which corresponds to the first stage of biofilm development.[33]

Biofilm formation at 27°C

Biofilm cells developed by *E. coli* on the different surfaces were also quantified at room temperature (27°C). The cells adhered to the 2 surfaces also showed varied results at various hours of incubation. At 24 hours, *E. coli* biofilm quantified on SS presented a count of 5.10 x 10^8 CFU/ml and 3.20 x 108 CFU/ml on TCP (Figure 2). The higher number of biofilm formed by *E. coli* on stainless steel than TCP in this work varied with the report of Stepanovic et al.,[34] that microorganisms form higher biofilm on hydrophobic surfaces such as plastic than hydrophilic surfaces such the stainless used for this study. The ability of *E. coli* to form biofilm on SS surface at room temperature is a great challenge to food industries, hospital and house hold environment as it is used widely for food storage and other packaging and transportation.

At 48 hours of incubation, enumeration of biofilm developed by *E. coli* on the two surfaces was more higher than at 24 hours and presented a count of 6.40 x 10^8 CFU/ml on SS while a count of 4.90 x10^8 CFU/ml was obtained on TCP (Figure 2). The adhesion of bacteria to surfaces occurs in two stages: reversible followed by irreversible adhesion.[35] During reversible adhesion, bacteria are easily removed by applying minimum force.[19] Irreversible adhesion initiates after 20 minutes to a maximum of 4 hours of contact at 4-20°C[36] and presents serious risks to the food industry, since the removal of irreversibly adhered cells is difficult and requires the application of strong mechanical force or chemical interruption of the adhesion using surfactants, sanifiers or heat.[37] Thus, there is a high probability that the irreversibly adhered cells will remain even after hygienization.[33] This is one of the main reasons for biofilm formation on surfaces in contact with food.

At 72 hours biofilm developed by *E. coli* on the tow surfaces was higher than the previous hours of incubation. The number of viable cells quantified on SS yielded a count of 9.00 x 10^8 CFU/ml which whilst a count of 6.40 x 10^8 CFU/ml was quantified on TCP. The increase in number of viable cells on the coupons was attributed to maturity of the biofilm cells leading to increase number of adhered cells. Mature biofilm formation occurs from 72 to 144 hours after initial adhesion, and may reach 240 hours.[38] Maturity occurs mainly through population density increase as well as by pronounced production and deposition of extracellular polymers, increasing biofilm thickness.[39]

Action of disinfectants against 168 hours biofilm at 10°C

After exposure to Hydrogen Peroxide (HP), the number of viable *E. coli* viable cells on SS was greatly reduced from 8.60 x10^8 CFU/ml (positive control) to 1.00 x 10^7 CFU/ml with log reduction value of 1.94, percentage survival fraction (% SF) of 1.16 while about 98. 84% were susceptible to the bactericidal effect of HP (Table 1). Compared to the positive control (5.80 x 10^8 CFU/ml) on TCP, the bactericidal action of HP has completely killed all the biofilm cells developed on TCP. The action of PAA, SH and PAA + SH at 10°C has also killed all the biofilm that was developed on TCP (Table 1). However, incomplete removal of the biofilm developed on SS was observed after treatment with PAA, SH and their combinations. Compared to the positive control on SS (8.60 x10 10^8), *E. coli* viable cells were reduced to 1.00 x 10^8 CFU/ml after treatment with PAA with LR value of 0.93 (Table 1). After the treatment with SH, the number of *E. coli* viable cells was reduced to 3.00 x10^7 CFU/ml with LR value of 1.03. The bactericidal effect of mixture of PAA + SH has reduced the number of *E. coli* viable cells to a count of 1.10 x 10^8 CFU/ml with an LR value of 0.89 (Table 1). Thus it can be seen that HP was most effective against the biofilm developed by *E. coli* followed by SH, then PAA while PAA +SH was the least although a good reduction of the biofilm was achieved with this combination. Although not similar strains, a work by Cabeca et al.,[23] reported SH to be effective against *Listria monocytogenes* biofilm developed on SS.

Because disinfectants are formulated to kill microbes, the treated carriers were expected to hold fewer viable cells than the untreated carrier. A small value for SF, a large value for LR and PK all indicate good disinfectant efficacy. The efficacy of hydrogen peroxide in killing most of the biofilm cells formed by *E. coli* on these surfaces might likely be attributed to low incubation temperature (10°C) which do not favor high biofilm formation. The resistance of *E. coli* to the tested disinfectants might also be attributed to the effect Extracellular Polymeric Substance (EPS) which usually surrounds the bacteria and decrease the penetration of antimicrobials into the cellular membrane which is the target site for most antibacterial and or antibiofilm agents. However, it was difficult to compare this work directly with other studies due to the variation in time and temperatures of incubation employed and the organism used.

Action of disinfectants against 168 hours biofilm at 27°C

At 27°C the bactericidal effect of the different disinfectants appeared to be less than it was at 10°C. After treatment with HP, the number of *E. coli* viable cells on stainless were reduced to 1.10 x 10^7 CFU/ml, with PAA action, the number was reduced to 1.20 x 10^8 CFU/ml. Less biofilm cells were killed after exposure to

the lethal effect of SH and mixture of PAA + SH with remaining surviving bacterial count of 1.70 x 10^8 CFU/ml and 1.30 x 10^8 CFU/ml respectively (Table 2). The bactericidal action of HP and mixture of PAA + SH was most effective and has completely killed all the biofilm cells developed on TCP without surviving bacteria, while after treatment with PAA the number of *E. coli* viable cells on TCP were reduced to 2.00 x 10^7 CFU/ml and 3.00 x10^7 CFU/ml with SH which appears to be less bactericidal unlike at 10°C where it was most effective (Table 2). It was found from this study that although the disinfectants have successfully reduced the biofilm developed on the surfaces, not all the biofilm cells were completely removed because of resistance mechanism. Bacterial resistance may originate from endogenous genes or may be acquired by mutation or the acquisition of plasmids or transposons.[40]

Once microorganisms have attached, they may become capable of withstanding normal disinfection processes. Incomplete removal of the biofilm will allow it to return quickly to its equilibrium state, causing a rebound in total counts following sanitization.[41] Surviving organisms rapidly create more extracellular polymers as a protective response to irritation by chemical cleaning agents and this leads to increasing resistance of microorganisms to antimicrobials. It has become clear that biofilm-grown cells express phenotypes distinct from the planktonic cells, one of their properties being increased resistance to antimicrobial agents.[42] Slow growth and/or induction of an *rpo S* - mediated stress response could contribute to biocide resistance.[43,44] The physical and/or chemical structure of exopolysaccharides or other aspects of biofilm architecture could also confer resistance by exclusion of biocides from the bacterial cells (Cabecal et al.,.[23] Finally, biofilm-grown bacteria might develop a biofilm specific biocide-resistant phenotype.[42]

CONCLUSION

In conclusion, the results of this work demonstrated that *E. coli* can develop high biofilm on stainless steel and Tissue culture at refrigeration and room temperature. Also, this study demonstrated that biofilm formation by *E. coli* increases with increased incubation periods.

Evaluation of the different disinfectants efficacy demonstrated that HP was a good disinfectant against the *E. coli* biofilm at 10°C followed by SH, and then PAA while mixture of PAA + SH was the least even though it has successfully reduced the biofilm cells on the surfaces. The combination of AA and SH does not produce a far better bactericidal effect than their individual actions. At 27°C HP was also the most effective followed by mixture PAA, then PAA + SH whilst SH was the least. Thus it can be concluded that HP and PAA can be good disinfectants agents against *E. coli* biofilm, since the biofilm was much developed on stainless steel at room temperature (27°C) and its elimination requires a strong bactericidal reaction and/ or effect. The mixture of PAA + SH had also proven to be a good disinfectant agent against *E. coli* biofilm and had produced a bactericidal effect greater than their Individual effect at room temperature and their combined effect at 10°C, while SH was the least at this temperature.

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