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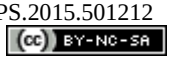
ISSN 2231-3354 

Assessment of Chemical Disinfectants Efficacy against *Escherichia coli* Biofilm Developed on Glass and Wood at Refrigeration and Room Temperatures

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ABSTRACT

Objectives: The aim of this work was to study the formation of biofilm on glass and wood coupons at refrigeration and room temperatures, different incubation periods and to assess the efficacy of hydrogen peroxide (HP), Para Acetic Acid (PAA), Sodium hypochlorite (SH) and mixture of PAA + SH against the biofilm.
Method: 200 µL of 10^8suspension *E. coli* ATCC 29922 was inoculated on the coupons inside petri dishes containing 20 ml of tryptic soy broth, incubated at 10 and 27^0C for 24, 48, 72 and 168 hours. Biofilm developed at each hour above was quantified by bead-vortex 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 effect were vortexed and enumerated by agar plating.
Results: The results showed that *E. coli* can develop high biofilm on wood apart from glass. After disinfection treatment, HP had the highest efficacy at 27°C followed by PAA then SH, whilst PAA + SH had the least.
Conclusion: It can be concluded that HP and PAA can be good disinfectants agents against *E. coli* biofilm.

INTRODUCTION

The term biofilm is used to describe matrix-enclosed bacterial population adherent to each other and/or to surfaces (Costerton *et al.,* 1995). Biofilm production is an important mechanism for bacterial survival and its occurrence together with antimicrobial resistance represent a challenge for clinical management. Biofilm formation occurs when microorganism attached to a surface and through growth and continuing colonization, spread over the surface. Biofilm formation occurs when microorganism attached to a surface and through growth and continuing colonization, spread over the surface. Bacteria in natural environment usually form biofilm communities of sessile organisms embedded in a hydrated matrix of extracellular polymeric slime with polysaccharides, nucleic acid and proteins (Costerton *et al.,* 1999). Inside the host, the matrix protects biofilm bacteria from exposure to innate immune defenses (such as opsonization and phagocytosis) and antibiotic treatments (Jesaitis *et al.,* 2003; Cerca *et al.,* 2007). *Escherichia coli* (*E. coli*) are genetically diverse species that causes diarrheal diseases and variety of extra intestinal infections which fulfill many or all of the proposed criteria for biofilm-associated infections (Kaper *et al.,* 2004). The diseases in which biofilms play a major role tend to be chronic and difficult to treat. In modern clinical microbiology, establishment of bacterial biofilm is considered a pathogenicity threat during chronic infections (Sritharan and Sritharan 2004). Biofilm in food processing environment is of special importance as it has the potential to act as the chronic source of microbial contamination that may lead to food spoilage or transmission of diseases (Stepanovic *et al.,*2004).The infectious dose of the pathogen is as low as 10 to 100 organisms/cells (Feng and Weagant 2002). *E. coli* infection is also responsible for most cases of hemolytic uremic syndrome, a major cause of acute renal failure in children (Boyce *et al.,* 1995). Infections due to pathogenic *E. coli* may be limited to colonization of mucosal surfaces or can

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disseminate throughout the body and had been reported to have effect in urinary tract infection, sepsis or meningitis and enteric diarrheal disease (Chen and Frankel, 2006). In food industry, pathogenic bacteria have been of considerable interest in the context of food safety and have provoked the interest of many research groups (Shi and Zhu, 2009). It is also evident that the attachment of pathogenic bacteria to food contact surfaces such as plastic, stainless steel, wood and glass and the subsequent biofilm formation are undesirable since the detachment of cells from the biofilm structure can lead to the cross - contamination of food products leading to food borne diseases (Brooks and Flint, 2008).

Microorganisms usually struggle to survive when exposed to a harmful environmental stress. When bacteria are exposed to sub-lethal levels of antimicrobials or biocides, only minor cell damage is caused and the consequences may include changes in their phenotype and induction of gene expression, giving rise to a more resistant population (Araujo *et al.*, 2011). Thus, the aim of this was to study the formation of biofilm by *E. coli* on glass and wood at refrigeration and room temperature (10 & 27^0C) and also to evaluate the efficacy of hydrogen peroxide, Para Acetic Acid, Sodium hypochlorite, and mixture of Para Acetic acid and Sodium hypochlorite against the biofilm.

MATERIALS AND 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 37^0C and stored at 5 ^{0}C for further experiment.

Preparation of Test Surfaces

Glass slides employed in this study were initially soaked in acetone for 1 hour to remove manufacturing debris, washed in detergent solution, rinsed twice with distilled water, air-dried and autoclaved together with wood coupons (3 cm x 1 cm) at 121^0C for 15 minutes prior to use (Chmielewski and Frank 2003).

Preparation of inoculum

One colony from the overnight cultures was inoculated into 50 ml plastic tube containing 5 ml of Tryptic Soy Broth (TSB) and was incubated at 37^0C for 2 hours. Following 2 hours of incubation, 2 ml of the incubated strain were inoculated into 200 ml of TSB in a 500 ml conical flask incubated in an orbital shaker at 37^0C for 16 hours (Chmielewski and Frank 2003) to allow the strain reach their exponential phase. After 16 hours of incubation, 10 ml of the incubated cultures 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). 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 (Merode *et al.*, 2006).

Biofilm formation in vitro

Biofilm formation on the two coupons was conducted using the method described by Kostaki *et al*., (2012). 200 µl of 10^8 CFU/ml suspension of *E. coli* strain was inoculated on each of the coupons inside petri-dishes and was 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 a period of 24, 48, 72 and 168 hours to allow for biofilm development.

Enumeration of biofilm cells

The enumeration of viable biofilm cells on glasses and wood coupons was performed after 24, 48, and 72 hours using bead-vortex method described by Giaouris and Nychas (2006). 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 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), vortexed for 2 min at maximum speed to detach biofilm cells from the coupons. Detached cells were subsequently enumerated by agar plating on TSA after ten-fold, six dilutions. Finally plates were removed after 24 hours of incubation. Developed colonies were counted taking a range of 3-300. The experiment was repeated three times and viable cells were expressed as colony forming unit per mill (CFU/ml).

Activities of Disinfectants against Biofilm

The disinfectants employed 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 separate petri dishes containing 20 ml of each of the disinfectants under study, incubated at 27ºC for 10 minutes with gentle shaking followed by deactivation with 10 ml of TSB for 5 minutes (Cabeca *et al.*, 2008). Positive controls treated the same way but with physiological saline. After 10 minutes, the actions of the disinfectants were deactivated by transferring the coupons into new petri dishes containing 10 ml of TSB and allowed to act for 5 minutes (D.I.F.C.O Manual 1984). Following 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 (Giaouris and Nychas 2006) in order to releases viable cells adhering to the coupons into the physiological saline. The control coupons were treated equally as the test control but with Physiological saline. To count viable cells, bacteria were enumerated by agar plating after ten-fold dilutions and incubated on Tryptic Soy Agar (TSA) at 37ºC for 24 hours. 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 which gives the survival fractions (SF), the percentage killed (PK) was evaluated using the formula: PK = (1-SF) x 100% while the log reduction (LR) factor was evaluated using the formula:

LR= Log_{10} (1/SF)……...(Hamilton, 2011).

RESULTS AND DISCUSSION

Biofilm Formation Assay at Refrigeration Temperature (10°C)

E. coli an important Gram-negative anaerobic bacterium which commonly contaminates food contact surfaces in food industries can develop biofilm on medically associated devices such as catheter and mechanical heart valves. It was found from m this work that biofilm developed on glass coupons varied with that developed on wood coupons. At refrigeration temperature enumeration of *E. coli* viable cells after 24 hours presented a count of 2.90 x 10^8 CFU/ml on glass while a count of 1.80 x 10^8 CFU/ml was obtained on wood which was less than the count obtained on glass (Figure 1).

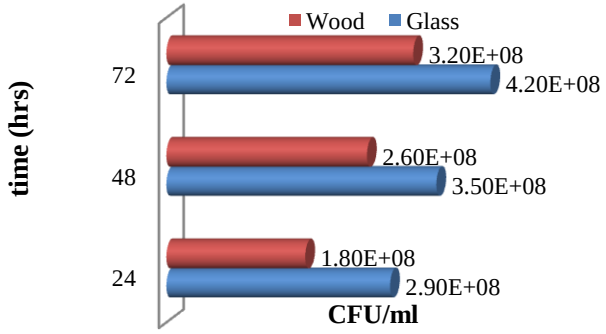

Fig. 1: Enumeration of *E. coli* Biofilm cells at Refrigeration Temperature (10 °C).

At 48 hours, the increase in incubation time has led to increase in number of biofilm cells developed on each of the surfaces. Enumeration of *E. coli* biofilm cells presented a count of 3.50 x 10^8 CFU/ml on glass which was relatively higher than 2.30 x 10^8 CFU/ml quantified on wood. Variation in biofilm density on the surfaces is mainly due to their hydrophobic nature. Fletcher and Leob (1979) noted that large number of bacteria attached to hydrophobic surfaces with little or no surfaces charge while moderate number attached to hydrophobic metals with positive or neutral charge and very few attached to hydrophilic negatively charged substrate. The increase in biofilm formation with increased incubation time in this study was in line with the work of Silagyi (2007) who reported increase in biofilm development by *E. coli* on glass steel from 6, 12 and 24 hours.

At 72 hours, biofilm development on the two surfaces was higher than the previous hours of incubation with resultant bacterial count of 4.20 x 10^8 CFU/ml and 3.20 x 10^8 CFU/ml on glass and wood respectively (Figure 1). The high biofilm formation by *E. coli* on glass in this work varied with the work of Adetunji and Odetokun (2012) who reported less biofilm formationon glass and higher biofilm formation on cement coupons by *E. coli*. The increase in biofilm formation with increase incubation time obtained in this work is similar to the result reported by Mahdavi *et al.,* (2008) who found that biofilm formed by *Salmonella enteritidis* on glass increased significantly from 2 to 20hours of incubation. However we are unable to find related biofilm published work on wood by *E. coli* to make comparison. Mature biofilm occur from 72 to 144 hours after initial adhesion, and may reach 240 hours (Heydon *et al.,* 2000). Biofilm maturity occurs through population density increase, pronounced production and deposition of extracellular polymers, as well as increasing biofilm thickness (Cheng *et al.,* 2007; Oliveira *et al.,* 2010).

Biofilm Formation Assay at Room Temperature (27 °C)

Enumeration of biofilm developed on the two surfaces by *E. coli* at room temperature showed that biofilm formation by the organism was more favored at room than at refrigeration temperature. After 24 hours enumeration biofilm formed by *E. coli* on glass yielded a count of 6.80 x 108 CFU/ml on glass and 3.90 x 108 CFU/ml on wood (Figure 2). The ability of *E. coli* to form biofilm on these surfaces at room temperature is a great challenge to food industries, hospital and house hold settings. This is because the increase use of wood in animal slaughter houses, chopping boards at home can provides adhesion sites for *E. coli* leading to biofilm formation and resultant outbreak of food borne diseases as well as other bacterial infections. One of the great biofilm formation issues in the food industry or other areas is cell detachment, which makes it a constant source of microorganism contamination in food, water, or new infection processes (Oliveira *et al.,* 2010).

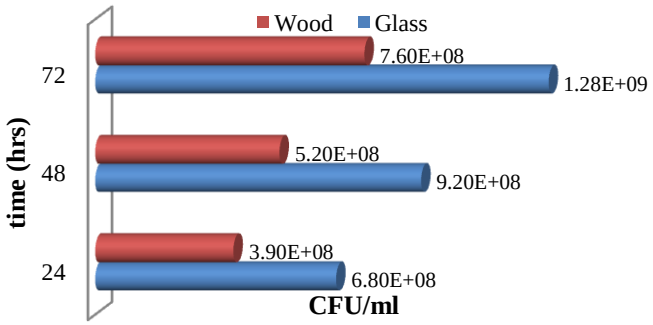

Fig. 2: Enumeration of *E. coli* Biofilm cells at Room Temperature (27^0C).

As observed after 48 hours of incubation, biofilm development on the surfaces also increases with increased incubation time. Following enumeration of *E. coli* viable cells, a count of 9.20 x 10^8 CFU/ml was obtained on glass while less biofilm with a count of 5.20 x 10^8 CFU/ml was obtained on wood (Figure 2). When the incubation time was extended to 72 hours, biofilm formation on the two surfaces developed much more on glass than the previous hours with a resultant bacterial count of 1.28 x 10^9 CFU/ml while a relatively higher biofilm cells with a count of 7.60 x108 CFU/ml was obtained on wood (Figure 2). The increase in high number of biofilm cells quantified on the surfaces especially on glass varied with the report of Di Bonaventura *et al.,* (2008) that bacteria usually attached to hydrophobic surfaces than hydrophilic, and increased hydrophobicity at high temperatures

such as 27 °C employed in this study may enhance the initial adherence of bacteria leading to a higher biofilm density.

Efficacy of Disinfectants at Refrigeration Temperature (10 °C)

The bactericidal effect of the various disinfectants at refrigeration temperatures produced varied results with some viable cells resisting the killing effects of the disinfectants. At refrigeration temperature following exposure to hydrogen peroxide (HP), *E. coli* biofilm cells developed on glass and wood were completely killed with no surviving cells. After treatment with acetic acid (PAA), the number of *E. coli* viable cells of the biofilm adhered to glass was reduced to 4.10 x 10^7less than the positive control (9.00 x 10^8 CFU/ml), with a log reduction (LR) value of 1.35 while the number of viable cells attached to wood was reduced to 3.00 x 10^7 CFU/ml which was also less than the positive control (7.00 x 10^8 CFU/ml) with LR value of 1.37 greater than that of glass (Table 1).

When the surfaces were treated with sodium hypochlorite (SH), the bactericidal action of the disinfectant has reduced the number of viable cells on glass to a count of 1.40 x 10^8 CFU/ml with LR value of 0.81 while the number of viable cells of the biofilm formed on wood was reduced to 1.00 x 10^8 CFU/ml with LR value of 0.85. Compared to the positive control on glass (9.00 x 10^8 CFU/ml) the action of PAA + SH has reduced the number of viable cells adhered to glass to count of 1.30 x 10^8 CFU/ml with LR value of 0.84 whilst the action of thesecombinations on wood has reduced the number of viable cells to a count of 3.00 x10^7 CFU/ml which was also less than the positive control (7.00 x 10^8 CFU/ml) as illustrated in table 1. It can be seen that at refrigeration temperature HP was the most effective against the biofilm on the surfaces, followed by PAA, then the mixture of PAA + SH while HP was the least effective against the biofilm although it has also reduced the viable cells to a greater extend (Table 1).

Although the LR values obtained in this work were not up to 3, greater number of viable cells of the biofilm developed on the surfaces was reduced. Many reports on disinfectants efficacy have proposed a LR value of 4 or 5 to be consider as effective. Sultan *et al.*,(2006) reported that in order to prove disinfectant efficiency, there has to be a 5-log reduction (a reduction in the number of microorganisms by 100,000-fold) in initial cell concentrations. Luppens *et al.,* (2002) reported that a disinfectant that resulted in more than a 4-log reduction in 5 minutes in a biofilm of cell concentration (4×10^7 to 1.3×10^8 CFU/cm^2) should be considered an effective agent on biofilms. Wirtanen *et al.,* (2002) proposed that for a biofilm test only a 3-log reduction was necessary, but Luppens *et al.,* (2002) pointed out that a 3-log reduction is too small for biofilms that can contain cells up to 1.3×10^8 CFU/cm^2 (Companac *et al.,* 2002; Sultan *et al.,* 2006).

Efficacy of Disinfectants at Room Temperature (27 °C)

At 27 °C compared with the positive control on glass (1.60 x 10^9 CFU/ml) the bactericidal effect of hydrogen peroxide on glass was not hundred percent effective with remaining surviving bacterial count of 7.00 x 10^7 CFU/ml and 1.36 LR values, while its action on wood has completely killed the biofilm (Table 2). After treatment of the biofilm on glass with PAA, a high number of viable cells resisted the bactericidal with surviving viable count of 1.00x 10^8 CFU/ml and LR values of 0.20 resulting in higher number of survival fraction (SF) of viable cells than the percentage killed (PK) (Table 2).The effect of PAA on biofilm developed on wood has reduced the viable cells of the biofilm to a count of 8.00 x 10^8 CFU/ml less than the cells on glass and with greater LR value of 1.16 than the LR value obtained on glass (Table 2).

High number of viable cells resisted the killing effect of SH on glass yielding a count of 2.30 x 10^8 CFU/ml with less LR value of 0.84. The action of SH on wood has to a greater extend reduced the viable cells of the biofilm to account of 1.80 x 10^8 CFU/ml, and with LR values of 0.97 greater than the values obtained on glass (Table 2). Hypochlorite is a very reactive compound and is generally known to be little effective against biofilms (Scher *et al.,* 2005). Increasing the concentration of the disinfectants beyond the recommended user concentration may increase the bactericidal activity, but such concentrations may not be relevant from a practical point of view because of the factors such as corrosion, solubility, health issues and costs (Moretro *et al.,* 2009). A study by Vestby *et al.,* (2010) also concluded that neither hypochlorite nor benzalkonium chloride achieved a 4log reduction, although when combining the disinfectants usedwith a synthetic furanone, this target was achieved (Corcoran *et al.,* 2014). In contrast and although not similar strain, Wong *et al.,* (2010) found that a range of disinfectants, including benzalkonium chloride and sodium hypochlorite, reduced 3-, 5-,and 7-day *Salmonella* biofilms by 4 log reduction.

Highest number of cells on glass resisted the killing effect after treatment of the biofilm with PAA + SH resulting in a count of 2.40 x 10^8 CFU/ml and with less LR values of 0.82. The effect of the mixture of the disinfectants on wood has reduced the viable cells of the biofilm to a count of 1.50 x 10^8 CFU/ml with LR values of 0.89 relatively greater than the LR values obtained on glass (Table 4). Preliminary data has suggested that covering a dried inoculum with disinfectant without any further mechanical action to improve contact between organisms and disinfectant, will usually result in lower reduction factors than those obtained with suspension test (Van Klingeren *et al.,* 1998). Thus the low LR values/factors obtained in this study may be attributed to not applying these mechanical actions. However, in many instances, the concentration necessary to reduce the cell numbers above the4 log threshold was also above the concentration recommended by the manufacturer (Wong *et al.,* 2010).

The difference in results may reflect the differences in the method of biofilm formation and in particular the surface area available for biofilm formation. Møretrø *et al.,* (2009) also highlighted the difference in efficacy of disinfectants using different methods of testing, such as the pellicle test, suspension test, and European surface test EN 13697:2001.

Table 1: Efficacy of Disinfectants against 168 hours Biofilm developed at 10 °C.

Disinfectants	Surfaces	Positive control (CFU/ml)	No. of surviving cells (CFU/ml)	LR	% SF	% Killed
Hydrogen peroxide	Glass	9.00×10^8	-	-	0.00	100
(HP)	Wood	7.10×10^8	-	-	0.00	100
Para Acetic Acid	Glass	9.00×10^8	4.00×10^7	1.35	4.44	95.56
(PAA)	Wood	7.10×10^8	3.00×10^7	1.37	4.23	95.77
Sodium hypochlorite	Glass	9.00×10^8	1.40×10^8	0.81	15.56	84.44
(SH)	Wood	7.10×10^8	1.00×10^8	0.85	14.08	85.92
PAA + SH	Glass	9.00×10^8	1.30×10^8	0.84	14.44	85.56
	Wood	7.10×10^8	3.00×10^7	1.37	4.23	95.77

Table 2: Efficacy of Disinfectants against 168 hours Biofilm developed at 27 °C.

Disinfectants	Surfaces	Positive control (CFU/ml)	No. of surviving cells (CFU/ml)	LR	% SF	% Killed
Hydrogen peroxide	Glass	1.60×10^9	7.00×10^7	1.36	4.38	95.62
(HP)	Wood	1.17×10^9	-	-	0.00	100
Para Acetic Acid	Glass	1.60×10^9	1.00×10^8	0.20	62.50	37.50
(PAA)	Wood	1.17×10^9	8.00×10^7	1.16	6.84	93.16
Sodium hypochlorite	Glass	1.60×10^9	2.30×10^8	0.81	14.38	85.62
(SH)	Wood	1.17×10^9	1.80×10^8	0.97	10.53	89.47
PAA + SH	Glass	1.60×10^9	2.40×10^8	0.82	15.00	85.00
	Wood	1.17×10^9	1.50×10^8	0.89	12.85	87.15

CONCLUSION

In conclusion, the results of this work demonstrated that *E. coli* can develop biofilm on wood with high density in addition to glass. This could be a serious problem in food industries were packaging, storing and transporting of food products were employed using these surfaces. It was also found from this work that *E. coli* biofilm formation increases with increased temperature and time. The bactericidal action of the different disinfectants has proved effective in reducing the biofilm cells at the two temperatures but HP has proven to be a good disinfectant agent than the rest of the tested disinfectants against *E. coli* biofilm at the two temperatures.

However, PAA has also proven to be a good anti biofilm agent than SH at 10 and 27 °C, while SH was more effective than PAA + SH at 27 °C. The mixture PAA + SH has not produce a more lethal effect than their individual effect. Thus there is the need to try different combinations of disinfectants to achieve synergistic action in biofilm elimination.

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