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Evaluation of Chemical Disinfectants Efficacy against *Escherichia coli* Biofilm Developed at 37°C on Glass and Stainless steel

Efikasi Disinfektan Kimia terhadap Pertumbuhan Biofilem Escherichia coli dipermukaan Kaca dan Keluli pada Suhu 37°C

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Abstract

This work aimed at studying biofilm development on glass and stainless steel (SS) at various incubation periods and assessing the action of hydrogen peroxide (HP), para acetic acid (PAA), sodium hypochlorite (SH), and mixture of PAA and SH against the biofilm. A 200 µl of 10^8 CFU/ml suspension of *E. coli* ATCC 29922 was inoculated on the coupons inside petri dishes containing 20 ml of Tryptic Soy Broth, incubated at 37°C for 24, 48, 72 and 168 hours. At each hour of incubation, viable cells developed were vortexed and quantified by agar plating. The action of the disinfectants against the biofilm was tested after 168 hours of incubation. The results showed that *E. coli* developed highest biofilm on glass than on SS coupons. After disinfection treatment, HP had the highest bactericidal effect with LR value of 1.29 on glass while SH had the least bactericidal effect with LR value of 0.81 on glass. It can be concluded from this work that HP can be a good disinfectant against *E. coli* biofilm than PAA and SH.

Key Words Biofilm, *E. coli*, Colony forming unit per mill (CFU/ml), Disinfectants, Log reduction (LR).

Abstrak

Kerja ini bertujuan mengkaji pertumbuhan lapisan biofilem pada kupon kaca dan keluli tahan karat (SS) bagi pelbagai tempoh inkubasi dan menilai tindakan hidrogen peroksida (HP), asid asetik (PAA), natrium hipoklorit (SH), dan campuran PAA dan SH terhadap biofilm. Sebanyak 200 µl 10^8 CFU / ml *E. coli* ATCC 29922 telah disuntik pada kupon dalam bekas petri yang mengandungi 20 ml sup Tryptic Soy dan dieram pada 37°C selama 24, 48, 72 dan 168 jam. Pada setiap jam pengeraman, sel-sel yang berdaya maju disedut dan dikuantitikan di atas plat agar dengan kaedah saduran. Tindakan disinfektan terhadap biofilem itu diuji selepas 168 jam pengeraman. Hasil kajian menunjukkan bahawa pertumbuhan filem E. coli adalah tertinggi pada kupon kaca berbanding kupon SS. Selepas rawatan pembasmian (disinfektan) didapati, HP mempunyai kesan yang tertinggi terhadap bakteria dengan nilai log pengurangan (LP) sebesar 1.29 pada kaca manakala SH mempunyai kesan yang terendah terhadap bakteria dengan nilai LP 0.81 pada kaca. Dapat disimpulkan dari kerja ini HP berkesan membasmi biofilem E.coli berbanding dengan PAA dan SH.

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Kata kunci Biofilm, *E. coli,* unit pembentuk koloni per mill (CFU/mL), Disinfektan, log pengurangan (PL).

INTRODUCTION

Biofilm can be defined as a population of microbial cells growing on a surface and enclosed in an armophous extracellular matrix (Donald, 2002). 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 adhered to a surface and through growth and continuing colonization, spread over the surface. According to a recent public announcement from the National Institutes of Health, more than 60% of infections including native valve endocarditis, otitis media, chronic bacterial prostitis, periodontitis and cystic fibrosis are caused by biofilms (Lewis, 2001, Donland and Costerton, 2002).

Escherichia coli (*E. coli*) had been an important Gram negative model for *in vitro* analysis of biofilm formation on abiotic surfaces (O' Toole *et al.,* 2000; Houdt and Michiels, 2005) because of the diseases it causes. *E. coli* are genetically diverse species that cause diarrheal diseases and variety of extraintestinal infections which fulfill many 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 because of the resistance to antimicrobials and/or disinfectants conferred to the organism by the extracellular matrix. The organism has emerged with increasing frequency as a food borne pathogen over the last 20 years and is responsible for causing serious illness and sequelae in susceptible humans (Bacon and Sofos, 2003). Outbreak of *E. coli* O157:H7 infections were found to be primarily associated with over- eating undercooked ground beef and a variety of other foods were also been implicated as vehicles (Ryu and Beuchat, 2005).

Microorganisms usually struggle to survive when exposed to extreme environmental stress, their survival paves the way for antimicrobial resistance. 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 (Araujo *et al.,* 2011). Some of the effects of biofilm resistance to antimicrobials include: delayed penetration of the antimicrobial compounds, alteration of cellular growth rate, and activation of general stress response, quorum sensing and induction of a biofilm phenotype. The aim of this work was to study the development of biofilm on glass and stainless steel (SS) coupons at various incubation times, and to assess its sensitivity against four different chemical disinfectants namely hydrogen peroxide (HP), para acetic acid (PAA), sodium hypochlorite (SH) and mixture of PAA and SH (PAA + SH).

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°C and stored at 5°C for further experiment.

Preparation of Test Surfaces

Glass slides and stainless coupons (3 cm × 1 cm) used for this study were initially placed 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 (Chmielewski and Frank 2003).

Preparation of Inoculum

The overnight cultures were used to make the inoculum. One colony from the overnight cultures was picked and inoculated into 5 ml of Tryptic Soy Broth (TSB) in a tube, 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 (Chmielewski and Frank 2003). Following 16 hours of incubation, 10 ml of the incubated cultures were centrifuged at 5,000 × *g* for 5 minutes at 10°C, washed twice in 10 ml of phosphate buffer saline (PBS) (pH 7.3) (Kostaki *et al.,* 2012). 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 carried out using the method described by Kostaki *et al*., (2012) with some modifications. 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 37°C for a period of 24, 48, 72 and 168 hours to allow for biofilm development.

Enumeration of Viable Cells

The enumeration of viable biofilm cells on the 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 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 minutes at maximum speed to detach biofilm cells from the coupons. Detached cells were subsequently enumerated by agar plating on TSA. Finally plates were removed after 24 hours of incubation. Developed colonies on the two surfaces were counted. The experiment was repeated three times and viable cells were expressed as colony forming unit per mill (CFU/ml) using the formula CFU/ml = No. of colonies developed x dilution factor /volume of culture plated.

Efficacy of Disinfectants against Biofilm

The efficacy of the disinfectants was tested after 168 hours. The disinfectants used for this study include hydrogen peroxide (HP) 30% (R and M, Essex, U.K), para acetic acid

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(PAA) (R and M, Essex, U.K). After 168 hours of 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 at 25 ± 2º C for 10 minutes with gentle shaking (Cabeca *et al.,* 2008). A positive control was performed by placing a coupon in a Petri dish containing 20 ml of sterile 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 were allowed to act for 5 minutes (DIFCO Laboratories, 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 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 ten-folds 6 dilutions with sterilized physiological saline and cultured on TSA at 37 ºC for 24 hours. Developed colonies were counted and transformed 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 × 100 and that gives the survival fractions (SF) (% resistance) while the percentage killed (PK) (% sensitivity) was evaluated using the formula: PK = (1-SF) × 100% while the log reduction (LR) was evaluated using the formula: LR = Log10(1/SF) (Hamilton, 2010).

RESULTS AND DISCUSSION

Biofilm formation at 24 hours

E. coli, a Gram-negative anaerobic bacterium which commonly contaminate food containers in food industries can develop biofilm on medically associated devices such as catheter and mechanical heart valves. It was found from this work that biofilm developed on glass coupons varies with that formed on stainless steel coupons. Enumeration of *E. coli* viable cells at 24 hours presented a count of 8.80 x 10^8 CFU/ml on glass and 5.80 × 10^8 CFU/ml on stainless steel as shown in Table 1.

Table 1 Enumeration of *E. coli* biofilm developed on the coupons at 24, 48 and 72 hours

Time (hours)	Glass (CFU/ml)	Stainless Steel (CFU/ml)
24	8.80×10^8	5.80×10^8
48	1.05×10^9	7.80×10^8
72	1.40×10^9	1.22×10^9

This shows that the number of cells adhered and formed biofilm on glass are higher than those on stainless steel at 24 hours. 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 (Oliveira *et al.,* 2010). The variation in biofilm cell density on glass than stainless steel in this study was in line with the work of Mahdavi *et al.,* (2008)

who reported high biofilm density on glass than stainless steel by *Salmonella enteritidis* after 20 hours of incubation. This is mainly attributed to the hydrophobic nature of the surfaces which do not favor strong or high biofilm formation because of weak adherence.

Biofilm Formation at 48 hour

At 48 hours the number of viable cells quantified on glass increased very much with a count of 1.05×10^8 CFU/ml while less viable cells with a count of 7.80×10^8 CFU/ml was obtained on stainless steel which was lower than that on glass (Table1). A study by Adetunje and Odetokun (2012) reported a high development of biofilm by *E. coli* on glass than on cement coupons. Bacterial attachment is influenced by cell surface and media as well as other environmental factors (Kumar and Anand 1998; Frank 2001). The high biofilm density formed on glass in this work varied with the work of Adetunje and Odetokun (2012) who reported higher biofilm development on cement coupons than on glass which they attributed to the high hydrophobicity of cement than glass. The increase in biofilm development by *E. coli* on the surfaces with increased incubation periods in this work was in line with the work of Silagyi (2007) who reported a strong biofilm development by *E. coli* on glass and stainless steel as incubation period was increased from 6, 12 and 24 hours.

Biofilm Formation at 72 hours

At 72 hours of incubation biofilm development also increased greatly with a huge viable cell count of 1.40×10^9 CFU/ml and 1.22×10^9 CFU/ml on glass and stainless steel respectively (Table 1). 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 may occur from 72 to 144 hours after initial adhesion, and may reach 240 hours (Heydron *et al.,* 2000). Maturity occurs mainly through increase of population density as well as by pronounced production and deposition of extracellular polymers, increasing biofilm thickness (Cheng *et al.,* 2007). Bacteria usually attached to hydrophobic surfaces than hydrophilic moreover, increased hydrophobicity at high temperatures such as 37° C, may enhance the initial adherence leading to a higher biofilm density (Di Bonaventura *et al.,* 2008). The high development of biofilm on these two surfaces could be a serious problem in food industries where packaging, storing and transportation of food products were employed using these surfaces.

Efficacy of Disinfectants against 168 hours Biofilm

The efficacy of disinfectants were tested after 168 hours of biofilm development on the two coupons in order to allow the organism (*E. coli*) to adhere strongly to the surfaces and develop a fully mature biofilm. Compared to positive control on glass (1.93×10^9 CFU/ml), the bactericidal effect of hydrogen peroxide has greatly reduced the number of biofilm cells with resultant surviving bacterial count of 1.00×10^8 CFU/ml and LR value of 1.29. About 5.18% bacterial cells resisted and survived the bactericidal effect while 94.82% were killed after exposure to this disinfectant (Table 2). Russell (2003) reported an evidence of a regulated adapted response in growing *E. coli* exposed to hydrogen peroxide,

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with the cells becoming resistant to normally lethal doses of peroxide and the synthesis of around 40 new proteins. The action of hydrogen peroxide on stainless steel has reduced the number of biofilm cells to a count of 1.50×10^8 CFU/ml with LR value of 1.06. The percentage of viable cells that survived and resisted the killing effect was 8.77% while 91.23% were killed (Table 2).

The bactericidal action of PAA on glass has reduced the biofilm cells to a count of 1.50×10^8 CFU/ml which was less than the positive control (1.93×10^9 CFU/ml), with LR value of 1.11 and percentage survival fraction (% SF) of 7.77% viable cells (Table 2). After treatment with PAA, the number of adhered viable cells developed on stainless steel was reduced to 1.90×10^8 CFU/ml less than the positive control (1.71×10^9 CFU/ml) with low LR value of 0.95 and % SF of 11.11% viable cells that resisted the bactericidal effect (Table 2).

The bactericidal effect of sodium hypochlorite on *E. coli* biofilm developed on glass has reduced the viable cells to a count of 3.00×10^8 CFU/ml which was less than the positive control (1.93×10^9 CFU/ml) with 0.81 LR value. The effect of this same disinfectant on stainless steel has reduced the viable cells developed on stainless steel from 1.79×10^9 CFU/ml to 1.20×10^8 CFU/ml (Table 2). The LR value of 1.15 on stainless steel was greater than that obtained on glass hence more viable cells were killed on stainless steel than on glass. The Percentage killed on glass was 84.46% while that of stainless steel was 92.98% with a much difference (Table 2).

Table 2 Number of survived cells after treatment with disinfectants

Disinfectants	Surfaces	Positive control (CFU/ml)	No. of surviving cells (CFU/ml)	LR	% SF	% Killed
Hydrogen peroxide	Glass	1.93×10^9	1.00×10^8	1.29	5.18	94.82
(HP)	S. steel	1.71×10^9	1.50×10^8	1.06	8.77	91.23
Para acetic acid	Glass	1.93×10^9	1.50×10^8	1.11	7.77	92.23
(PAA)	S. steel	1.71×10^9	1.90×10^8	0.95	11.11	88.89
Sodium hypochlorite	Glass	1.93×10^9	3.00×10^8	0.81	15.54	84.46
(SH)	S. steel	1.71×10^9	1.20×10^8	1.15	7.02	92.98
PAA + SH	Glass	1.93×10^9	1.10×10^8	1.24	5.70	94.30
	S. steel	1.71×10^9	1.80×10^8	0.93	10.53	89.47

CFU/ml = colony forming unit per mill, LR = log reduction, % SF = percentage survival fraction, % Killed = percentage killed

It was found from this work that exposure of *E. coli* biofilm viable cells developed on the surfaces to mixture of PAA and SH yielded closely similar results. After treatment with HP + PAA on glass, the number of viable cells was reduced to 1.10×10^8 CFU/ml far less than the positive control (1.93×10^9 CFU/ml) with LR value of 1.24 greater than the LR values obtained when treated with only PAA and SH (Table 2). The bactericidal effect of the mixed disinfectants on stainless steel had reduced the number of *E. coli* viable cells from 1.71×10^9 CFU/ml (positive control) to 1.80×10^8 CFU/ml with low LR value of 0.98 (Table 2). It was noted that the bactericidal effect of HP + PAA was higher than that of their individual effect but less than that of HP. Although the strain were different, the good

action of PAA and SH on stainless steel in this work coincided with the work of Cabeca *et al.,* (2006) who reported a good bactericidal effect of PA which reduced the number of *Listeria monocytogenes* biofilm viable cells from 6.3 log CFU/cm^2 to 1.1 log CFU/cm^2 while SH to1.0 log CFU/cm^2.

However, 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 (Sultan *et al.,* 2006). 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 as an effective agent on biofilms. Wirtanen *et al.,* (2003) 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 up cells to 1.3×10^8 CFU/cm^2 (Companac *et al.,* 2002; Sultan *et al.,* 2006). Although the LR values of obtained in this work were less than the values reported by Luppens *et al.,* (2002) the disinfectants had also proven a good disinfectant efficacy. To achieve a good disinfection efficacy, there is a need to apply mechanical action such as brushing, wiping or mopping which result in better contact between microorganisms and disinfectants. 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 (Klingeren *et al.,* 1998). Thus the low LR values/factors obtained in this study may be attributed to not applying these mechanical actions.

It be noted that we have not use paired isolates as sample during our laboratory work, and as such we cannot run an ANOVA test for our results, since the conditions to run the test were not fulfilled.

CONCLUSION

In conclusion, the result of this work demonstrates that *E. coli* can develop biofilm on glass with high density than on stainless steel. It was also found that biofilm development increased with increase in incubation time. The action of the different disinfectants has proven effective in reducing the biofilm cells but HP had proven to be a good disinfectant agent than PAA and SH. The Mixture of PAA + SH had also proven to have bactericidal effect greater than their individual effect against *E. coli* biofilm even though none of the disinfectants kills the viable cells completely.

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