Journal of Applied Pharmaceutical Science Vol. 5 (10), pp. 118-125, October, 2015
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2015.501020
ISSN 2231-3354

Evaluation of biofilm formation and chemical sensitivity of *Salmonella typhimurium* on plastic surface

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ARTICLE INFO

Article history:
Received on: 23/08/2015
Revised on: 11/09/2015
Accepted on: 24/09/2015
Available online: 28/10/2015

Key words:
Biofilm. *S. typhimurium*, Plastic surface, Disinfectant, Treatment.

ABSTRACT

S. typhimurium is an important socioeconomic problem in several countries, mainly in developing countries where it is reported as the main responsible for the food-borne disease outbreaks. A biofilm can be explained as a group of cells, diverse species or mono-species that are fixed to a surface and/or to one another. This study aimed to evaluate the biofilm formation of *S. typhimurium* on the plastic surface as well as to determine the relationship between contact time and incubation temperature. Crystal violet assay was performed to quantify the biofilm formation with and without treatments based on the value of optical density at 600nm of the destaining crystal violet at different interval of time. The outcomes of the result indicated that, the attachment of bacterial cells to the plastic surfaces increased with the increased contact time and determined by temperature. The values of OD600 at 37 °C for 24, 48 and 72 hours were 0.770, 0.968 and 2.363 respectively. This indicated that, the formation of biofilm by *S. typhimurium* on plastic surfaces varied with contact time. For the disinfectant treatments, hydrogen peroxide with 91 % sensitivity was the highest in treatment of *S. typhimurium* cells, followed by the mixture of sodium hypochloride and paracetic acid with 70 %, then paracetic acid with 67 %. Considering this result, *S. typhimurium* formed a biofilm on the plastic surface, hygienic activities on a plastic surface in food industry during handling, processing, distribution and storage of food should be a concerned and these disinfectants are suggested for the treatment of *S. typhimurium*.

INTRODUCTION

Food is very important to our life, but once the food has been grown and harvested, it gets relocated to various conveniences that help to prepare and package the food for domestic purpose, or in most cases for commercial consumption. There are different modern machines that make the routine items we find on supermarket shelves a suitable reality. These machines do everything from the heat seal to blister pack and from bottling to canning, all the while relying on plastics that can take the constant wear and tear of the speed and weight of all the food items being moved and prepared. Plastics also resist the processes that go into maintaining modern high speed equipment and also bring added safety measures to make sure our food supply is safe to eat. Nevertheless contamination usually occurs during all this process which at the end leads to serious disease outbreaks in our communities (Autunes *et al*., 2003). *S. typhimurium* is a bacterium, that is zoonotic in nature, and pathogenic to human, that is commonly found specifically in the gut and intestinal tract of animals including both wild and domestic animals. Subsequently, there are various courses that permit its passage into the food chain. At the level of processing of raw meat, the digestive tracts that were already colonized with *Salmonella* can easily get contact with the rest of the meat and possibly causes a cross contamination of the whole surfaces or items around.

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Salmonella can likewise get spread on vegetables and fruits through the fecal substance already contaminated in fresh water and soil (Stepanovic *et al*., 2004) It is one of the most problematic zoonosis in terms of public health all over the world because of it's high endemicity, but mainly because of the difficulty in controlling it (Autunes *et al*., 2003). *S.* Typhimurium had become a global public concern for health, because of the antibiotic resistance and extensive host range (Autunes *et al*., 2003). Although to be more virulent to date, factors relevant to its weakness are not yet overcome until now. Much research on the formation of biofilm by this strain on different surfaces need to be considered and verify, in order to understand its mechanisms, so that a solution possibly can be put in place for these pathogenic bacteria (Gilbert *et al*., 2003). These pathogenic Gram-negative bacteria popularly called *Salmonella* serovar Typhimurium, causes food-borne diseases commonly known as the gastroenteritis in a human being. Withhold the modem mortality and morbidity of the weekly detail report from CDC for Surveillance of foodborne disease outbreaks, *Salmonella* leads to the most outbreaks-related hospitalizations (CDS, 2013). *S. typhimurium* has endured as one of the most commonly isolated serotypes withhold to the yearly report on the *Salmonella* serovar from the National enteric diseases observation in 2011. *S. typhimurium* continues to cause Nationwide outbreaks that are in connection with contaminated food of poultry in 2011, ground beef in 2011, peanut butter in 2009 and tomatoes in 2000 (Maki and Denis, 2009).

A biofilm can be explained as a group of cells, diverse species or mono-species that are fixed to a surface and/or to one another. Biofilm cells produce proteinaceous substances that permit synergic development and shield from conceivable harsh situations it may experience within the environment. Van Leeuwenhoek a Dutch researcher in the seventeenth century was the first individual to find biofilm cells on his dental plaque, which he called as "animacules" (Danlan, 2002). This disclosed study was further explored and portrayed in 1978 when it was observed that the microorganisms inside a biofilm develop inside a lattice that permits them to join hard to a surface and act distinctively to their planktonic counterparts (Danlan and Costerton, 2002). At the point when examining the ecosystem of the environment with the microscopy, it was reasoned that 99.9% of the microorganisms evaluated developed in a biofilm on a scope of surfaces (Danlan and Costerton, 2002). Immediately when biofilm cells were formed on a new surface around our environment, especially in food processing areas they are not only capable of causing cross contaminations on the various materials within the food preparation areas or food equipment, but also the effect of this contamination may result in a potential health hazard to the consumers of that product (Maki and Dennis, 2009). The main objectives of this study were consequently to evaluate the biofilm formation potentials of *S. typhimurium* cells on plastic surfaces, quantify the biofilm cells formed on the surface and determine the relationship between different incubation time and temperature. And finally, test for the sensitivity of *S. typhimurium* cells on some chemical disinfectants treatment (Maki and Dennis, 2009).

MATERIALS AND METHODS

Preparation of Inoculum

The overnight cultured of *S. typhimurium* 14028 was inoculated by taking a single colony in 5 ml tryptic soy broth (TSB) and incubated at 37 ^{o}C for 1 h. The culture was then put into a conical flask containing 200 mL of the TSB, and incubated at 37 ^{o}C for 16 h inside a rotary shaker. The cells were harvested by centrifugation at (6000rpm, 5 min, 10 ^{o}C). The pellet was washed twice with phosphate buffer saline (PBS) and re-suspended in 10 mL of TSB. 1 mL of the suspension was used to measure the optical density (OD) which was 0.5 at 600 nm, which is equivalent to 10^{8} $CFUmL^{-1}$. This suspension was used in this study as the inoculum (Van Merode, 2006; Kostaki *et al*., 2012).

Test surface

The test surface is a Micro titer plate (96 Wells) which was used as a plastic surface in the study. Sterile microplates were cleaned and made ready for the experiments (Chmielewski and Frank, 2003).

Development of Biofilm on Microplates (plastic)

To developed biofilm on plastic, microplate was used. 200 µL of the inoculum (10^{8} $CFUmL^{-1}$) was dispensed into some selected sterile 96-wells PS Microtiter plates, in replicates, 200 µL of PBS as control was also used in separate wells and incubated at 28^{o}C for 3 hours following the process of attachments (on the pirates surfaces of the wells). After the attachment hours, the planktonic cells were removed from the wells (through the use of multichannel pipette). All the wells were washed twice with phosphate buffer saline (PBS) in order to remove the loosely attached cells, 200 µL of the TSB was added to each well. This process of inoculation and supplement of growth nutrients was conducted on to the different sets of PS Microplates based on the designed of the study. Each PS Microplates were incubated under its respective temperature (10, 28 and 37 ^{o}C) and time for the sets of 24, 48 and 72 hours respectively. These enable the attached bacteria to replicate and formed biofilms. At each interval of 24 hours growth nutrient in each well were removed, washed twice with PBS and subsequently the TSB were renewed. After the formation of biofilm, the media were removed and the wells were washed, stain with 200 µL of 1% crystal violet solution to each well, then left at room temperature to get stained. Each plate was washed with 200 µL of deionized water to remove the excess stain. The stained wells were being solubilized with 200 µL of ethanol-acetone mixture (80/20, Vol/Vol). A microplate reader (ELIZA infinity pro 200) was used to observed the dye absorbance of the suspended biofilm cells at 600nm (A_{600}), (Van Merode *et al*., 2006).

Crystal Violet Stain and Biofilm Quantification.

Crystal violet assay was the method used to quantify the biofilm formation of *S. typhimurium* cells formed on plastic surfaces. This assay was adapted from Kostaki *et al.,* (2012) and

Pui *et al*., (2011a) with some modifications. After the total incubation time had reached, each microplate were washed and rinsed three times using 200 µL of PBS, in order to remove the loosely attached cells. Then air dried and the attached bacterial cells were stained with 200 µL of 1% (w/v) crystal violet (CV. Merck, Darmstadt, Germany) for 20 minutes. And the stained were removed from the wells and rinsed with 200 µL of deionized water and air dried. The attached stained together with the bacterial cells were solubilized with 200 µL ethanol-acetone mixture (80/20, Vol/Vol) for 20 minutes. The suspension was used to measure the optical density (OD) at 600 nm, with Microplate reader (ELIZA infinity pro 200) (Kostaki *et al*., 2012; Pui *et al*., 2011b; Van Merode *et al*., 2006).

Disinfectant Treatments

Different research techniques were being used in disinfectants treatments of biofilm formed on different surfaces. In this study the method used by Kostaki *et al*., (2012) and Pui *et al*., (2011a) was adopted with some slight modifications. The surface used was allowed to reach the 7th day of incubation time for a mature biofilm to be formed. Growth media were carefully renewed at every 24 hour by the used of multichannel pipette, after twice washing of the wells with 200 µL of PBS (each time) through which the loosely attached cells were being washed. After a total of 168 hours, wells were washed and 200 µL of each disinfectant was dispensed into the respective wells. Four different disinfectants were used in this research (Sodium Hypochlorite (NaOCl), Paracetic acid ($C_2H_4O_3$), Hydrogen peroxide (H_2O_2), and a mixture of NaOCl and Paracetic acid ($C_2H_4O_3$). The disinfection treatments were carried out at 15^{o}C for 6 minutes. This was done in order to imitate the conditions encountered in the food industries. After disinfection, the disinfectants were replaced with 200 µL of TSB to each well, which deactivated the disinfectant action after 6 minutes and removed after a minute. Then air dried and the attached bacterial cells were stained with 200 µL of 1 % crystal violet (CV. Merck, Darmstadt, Germany) for 20 minutes. And the stained were removed from the wells by rinsed process with 200 µL of deionized water and air dried. The attached stained together with the bacterial cells were solubilized with 200 µL ethanol-acetone mixture (80/20, Vol/Vol) for 20 minutes. The suspension was used to measure the optical density (OD) at 600 nm, with Microplate reader (ELIZA infinity pro 200) (Kostaki *et al*., 2012; Pui *et al*., 2011b; Van Merode, 2006).

RESULT AND DICUSSION

Biofilm formations on surfaces have different methods through which different surfaces are capable of supporting it formation. Some researchers thought of surface that are more useful in our environment while others think of surfaces that are necessary to be used in our daily life activities and explore them. In this study 96 wells microplate was also used to developed biofilm, so that a tangible justification can be made on the plastic itself. The quantification of biofilm that were formed on the plastic surface (microplate) was conducted by the used of ELIZA reader (infinity pro 200) after stained with a crystal violet. The optical density (OD) readings are observed at 600nm (A_{600}), and the result was indicated in Table 1.

Table 1: Biofilm formation of *S. typhimurium* on microplate (plastic) at different incubation temperature and time, represented by optical density(OD 600nm)

TIME (h)	MEAN VALUE(OD) (600 nm)		
	10^{o}C	28^{o}C	37^{o}C
24	0.164944	0.209800	0.770222
48	0.219044	0.577567	0.967756
72	0.966978	1.993511	2.363133

Table 2: Disinfectant of seven (7) Days Biofilm formed on Plastic (OD) Observed.

DISNT	TEM (^{O}C)	UNTRD (CFU/mL)	TRETD (CFU/mL)	RES (%)	SEN (%)
H_2O_2	10	1.02113	0.10983	10.75	89.25
	28	1.30653	0.12756	9.76	90.24
	37	3.14053	0.21950	6.45	93.55
$C_2H_4O_3$	10	1.07123	0.35583	20.78	79.22
	28	1.25453	0.66016	52.62	47.38
	37	1.92865	0.69450	36.00	64.00
NaOCl	10	0.94930	0.84826	89.35	10.65
	28	1.84873	1.12533	60.87	39.13
	37	1.36763	1.01620	74.30	25.70
$C_2H_4O_3$&NaOCl	10	1.37770	0.31726	23.02	76.98
	28	1.67950	0.53490	31.85	68.15
	37	1.78920	0.64232	35.89	64.11

Biofilm formation of *S. typhimurium* was conducted on PS microplates under different incubation temperature (10, 28 and 37 ^{o}C) and time 24, 48 and 72 hours as indicated in Figure 1, 2, 3 and 4 respectively, *S. typhimurium* formed biofilm on plastic surface in all the sets of temperature used. For all the tested sets of hours used 72 h as the longest incubation time produced 2.36 as the highest OD value of biofilm density of *S. typhimurium* at 37 ^{o}C, followed by 1.99 under 28 ^{o}C as the second highest OD value and then, very closed to this two OD values was 0.97 under 37 ^{o}C at 48 h of incubation time. While 0.16 OD value under 10 ^{o}C at 28 h was the lowest of all the OD of biofilm density of *S. typhimurium* produced on PS microplates. The plastic surface used in this study was able to formed biofilm cells on each of the wells, which gradually aggregate and formed a mature community under a different level of temperatures used. This was reported in previous studies that microorganisms, usually got attached to hydrophobic surfaces easily than in hydrophilic surfaces (Bendinger *et al*., 1993; Danlan, 2002). In other studies conducted on a different strain of *S. typhimurium* indicated that, all of the strains have the ability to formed biofilm on plastic microplates (Stepanovic *et al.,* 2004). From the incubation time at different incubation temperature tested for *S. typhimurium* on a plastic surface supported 0 biofilm cells density formation at 0 hours. This clearly indicated that, there was no any biofilm cells formation by *S. typhimurium* on the plastic surface at 0 h. Biofilm formation comprises of stages before establishment on a surface (i.e. planktonic stage, attachment, colonization & dispersal stage), this may be a reason why *S. typhimurium* cells did not form any

biofilm at zero time, because individual cells need time to become adapted to new environmental conditions immediately they are introduced (inoculated) in a fresh surface (Pui *et al*., 2011a).

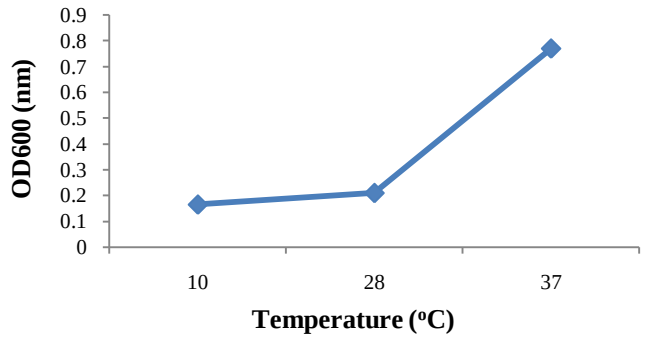

Fig. 1: Mean value of biofilm formation by *S.* Typhimurium on plastic surface, after 24 hours of incubation time at (10, 28 and 37 °C), represented by OD_{600}. Each bar represents the standard error mean of the triplicate measurements.

Biofilm formation on microplates initially begins from 0 to 0.16 after 24 h at 10 °C (Figure 1) , which later continue to increase to 0.22 at 48 h (Figure 2), this OD value continued to increase significantly at 72 h up to 0.97 (Figure 3).

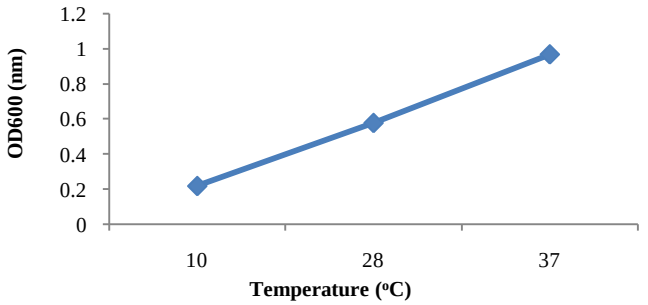

Fig. 2: Mean value of biofilm formation by *S. typhimurium* on plastic surface, after 48 hours of incubation time at (10, 28 and 37) °C, represented by OD_{600}. Each bar represents the standard error mean of the triplicate measurements.

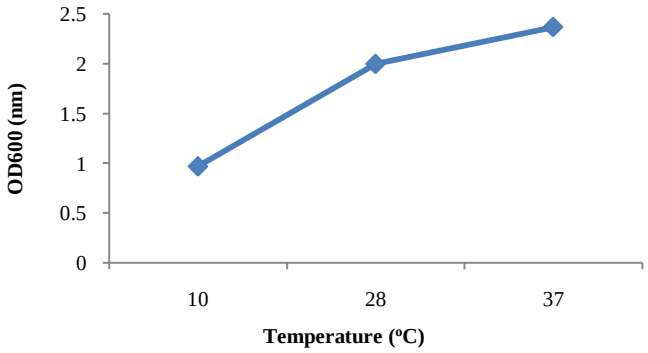

Fig. 3: Mean value of biofilm formation by *S. typhimurium* on plastic surface, after 72 hours of incubation time at (10, 28 and 37) °C, represented by OD600. Each bar represents the standard error mean of the triplicate measurements.

Similarly under 28 °C incubation temperature the value of OD begins from 0 to 0.22 and increased with an increased time at 48 h to 0.58 which continue to increase up to 1.99 OD value at the last incubation time (72 h) used in this study reached. 37 °C was the third incubation temperature used, and the value of biofilm density begins to formed from 0 to 0.77 OD value which increase to 0.97 at 48 h and continue to increase with an increase incubation time to 2.36 OD value at 72 h. Considering the outcome of this result indicate that biofilm formation increased with an increased in incubation time and temperature and high temperature favored the growth of *S. typhimurium* cells. This result indicated that, the attachment ability of *S. typhimurium* cells on a plastic surface is directly related to the amount of incubation time used. When the incubation time was increasing the bacterial cells have virtually more enough time to produce more biofilm cells. And on each plastic surface that supported a greater densities of biofilm cells of *S. typhimurium*, also indicated the greatest interaction forces between the cells and the plastic surface. Thus proven and reported that, the attachment ability of *S. typhimurium* cells on a fresh surface always increase with an increased contact time (Ukuku and Fett, 2002).

A bacterial cell produces polysaccharides, which provide a suitable means of coordination and different defensive mechanisms to the environmental factors. From this point, bacterial cells begin the formation of biofilm cells through collective efforts of different cells which later leads to the maturity of the community (biofilm) (Van Houdt and Michiels, 2010). From the outcome of this study, we observed that, the OD values gradually increased with an increased incubation time. A continuous washing of the Microplates (96 wells) wells were conducted twice at each interval of 24 hours of incubation, but as long as the incubation time was increased the biofilm cells form on the plastic surface are not easy to be remove or rinsed off, during this simple washing process. This was also an evidence reported by different past researchers like, the one conducted by Pui *et al*., (2011a), the OD values obtained in this study indicated that, the number of attached biofilm cells on a plastic surface increased over an increased incubation time, and 72 hours was the incubation time at which the highest density of attached biofilm cells were formed (Pui *et al*., 2011a). Biofilm formation conducted in this study was quantified using crystal violet method, through which the suspension obtained from the crystal violet stained were used to measure the OD values of different wells in the plastic surface. But considering the OD values generated may be affected by variable hydrodynamic shear, which normally occurs due to changes in the flow rate of the suspension. The attached biofilm cells dislodge more easily when the flow rate changes within a system (Hall-Stoodley and Stoodley, 2005). Also OD values are affected by the crystal violet stain, because during the process of staining of the biofilm cells, a lot of crystal violet dye gets deposited within the pirates of the wells which will eventually overestimate the number of adherent bacterial cells of the biofilm (Merritt *et al*., 2005). Different properties of the attachment surfaces are very important factors, which usually determine the biofilm formation potential. These properties include the surface roughness, disinfectability, clearability and vulnerability etc. all this properties influence the ability of bacterial cells to adhere on to a particular surface (Van Houdt and Michiels, 2010).

Temperature is the second important environmental factor that determines the bacterial growth after the media. As indicated in figure 4, in this study the growth of *S. typhimurium* cells were influenced by temperature, the density of the cell were much formed under a high temperature used. In all the sets of incubation temperature used, 37 ^{o}C supported the highest densities of *S. typhimurium* biofilm cells on a plastic surface, and then followed by 28 ^{o}C incubation temperature. The outcome of this study was similar to the report made that; *S. typhimurium* formed higher density of biofilm cells at 37 ^{o}C incubation temperature (Stepanovic *et al*., 2004). This was how biofilm cells of *S. typhimurium* were formed on a plastic surface with a continuous increasing density, from 10 ^{o}C, to 28 ^{o}C up to 37 ^{o}C incubation temperatures. Different studies were reported that, the formation of biofilm cells increased with and increased temperature (Mai and Conner, 2007). Crystal violet method used in this study was a very common technique used in the detection and quantification of the biofilm cells. This method is a very easy and convenient way to observed microorganisms in their habitat which provide many researchers with a lot of access to examine the formation of biofilm cells (Pui *et al.*, 2011b). This method of quantification involved the process of washing, staining, decolourization and observation under spectrophotometer for easy determination of optical density of the formed biofilm cells (Chavant *et al*., 2007; Oh *et al*., 2007). This method, also provides the ability to quantify, detect and differentiate many gram positive and gram negative bacteria. But nevertheless it comprises of some little error, because along the process the values of the OD obtained, contained not only the biofilm cells, but also many remaining dust of the stain used which are already attached with the bacterial cells and cannot be easily differentiated. Therefore, many studies that used this method will end up with OD values that are mixed with a stain and bacterial cells (Pui *et al*., 2011b).

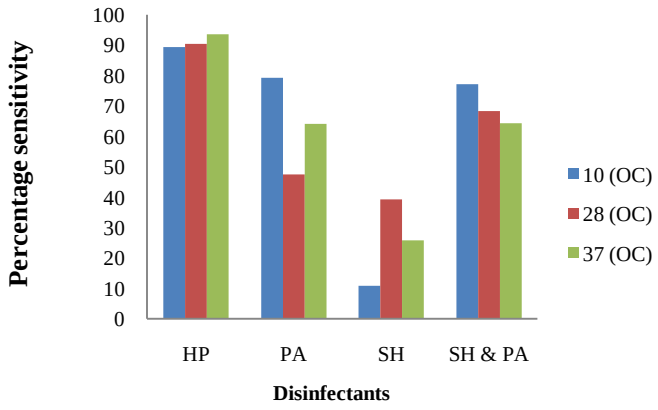

Fig. 5: Indicated the percentage sensitivity (Percentage of killed) biofilm cells of *S. typhimurium* on plastic surfaces following a 6 minutes exposure to 200 µL of hydrogen peroxide (H_2O_2), Paracetic acid, Sodium Hypochloride and a mixture of Paracetic acid & Sodium Hypochloride ($C_2H_4O_3$/NaOCl) disinfectants. The cultured biofilm cells were left to form on the plastic surface used for 7 days (168h) in a TSB at three different temperatures (10, 28 and 37 ^{o}C) with a renewal of medium at every 24 h).

Disinfectant treatment was conducted on biofilm cells of *S.* Typhimurioum formed on plastic surface after 7 days (168h) time of incubation under three different sets of incubation temperatures (10, 28, and 37 ^{o}C). Four different sets of disinfectants were used in the treatment process, Hydrogen Peroxide (H_2O_2), Paracetic acid ($C_2H_4O_3$), Sodium Hypochloride (NaOCl) and a mixture of Paracetic acid & Sodium Hypochloride respectively. The formed biofilm cells of *S. typhimurium* were exposed in to 200 µL of each disinfectant for 6 minutes in the microplates, in order to mimic the situations taking place in the food industries during treatment processes. Among all the four sets of disinfectant used Hydrogen Peroxide indicated a very high sensitivity of the formed biofilm cells of *S. typhimurium* by killing 89 % under 10 ^{o}C, 90% under 28 ^{o}C and 94 % under 37 ^{o}C incubation temperatures. Followed by the mixture of Paracetic acid and Sodium Hypochloride which killed 77 % under 10 ^{o}C, 68 % under 28 ^{o}C and 64 % under 37 ^{o}C incubation temperatures. The third effective disinfectant was paracetic acid which killed 79 % under 10 ^{o}C, 47 % under 2 8^{o}C and 64 % under 37 ^{o}C incubation temperatures. Sodium Hypochloride indicated the lowest sensitivity on the biofilm cells of *S. typhimurium*, and this made it to be less effective in the treatment process conducted. After the treatment process of biofilm cells of *S. typhimurium* had formed on the plastic surface, the number of the viable cells obtained from each disinfectant treatment indicated in figure 6. From the outcome of the result, sodium hypochloride with 89 % under 10 ^{o}C, 61 % under 28 ^{o}C and 74 % under 37 ^{o}C incubation temperature are the percentage of the number of viable cells after treatment, indicated to be the lowest effective disinfectant in the treatment of the formed biofilm cells of *S. typhimurium*. Then, followed by paracetic acid with only a single high number of viable cells percentage of 53 % under 28^{o}C incubation temperature.

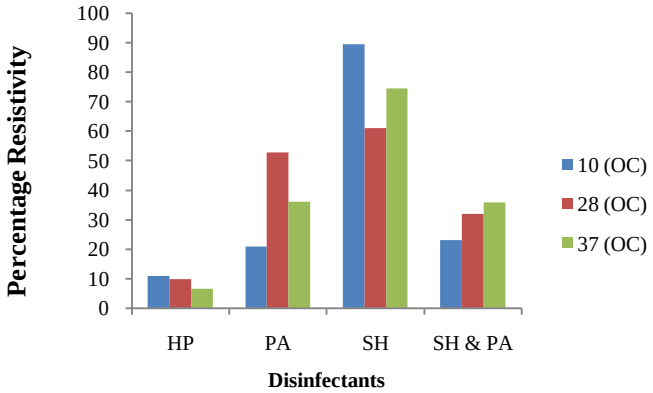

Fig. 6: Indicated the percentage of the resisted (viable cells) *S. typhimurium* biofilm cells on a plastic surfaces following a 6 minutes exposure to 200 µL of hydrogen peroxide (H_2O_2), Paracetic acid, Sodium Hypochloride and a mixture of Paracetic acid & Sodium Hypochloride ($C_2H_4O_3$/NaOCl) disinfectants each. The cultured biofilm cells were left to form on the plastic surface used for 7 days.

Disinfectant Treatments

The result obtained in this study for the disinfection treatment of *S. typhimurium* biofilm cells formed on surfaces after

a total of 7 days (168) hours were conducted with four different sets of disinfectants which were presented in figure: 5 and 6, and summarized in figure 7 indicated that, the biofilm cells of *S. typhimurium* are to a large extent sensitive to disinfectants and to a very small extent resistant to disinfectants used in this study. This process was fully observed based on the outcome displayed by the various disinfectants used in the treatments on biofilm cells formed on plastic surfaces at different incubation time and temperatures. The potential of any formed biofilm on a surface varied, mostly, depending on the nature of the surfaces used and also the resistivity shown by bacterial cells at the level of treatment with disinfectant as well, varied significantly depending on the surface used. All the biofilm cells formed on the plastic surface used in this study indicated a very high sensitivity to hydrogen peroxide with a maximum percentage. This result reflected a report made that, hydrogen peroxide as a disinfectant solution contained about 7.5% hydrogen peroxide, had been selected and approved by the USFDA, for the process of high-level sterilization and disinfection in health care settings (HAIs, 2015).

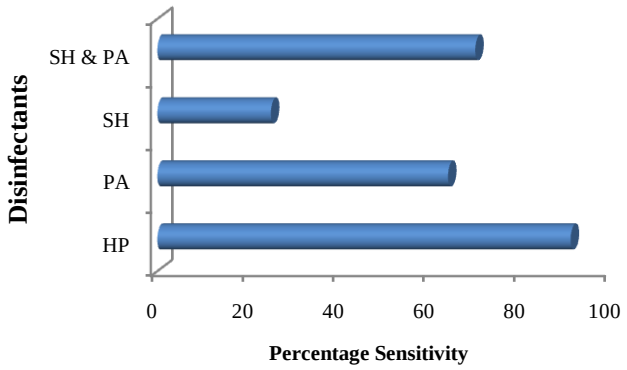

Fig. 7: Total percentage killed biofilms cells of *S. typhimurium* following a six (6) minutes exposure to (hydrogen peroxide (H_2O_2), paracetic acid ($C_2H_4O_3$), sodium hypochloride (NaOCl) and a mixture of sodium hypochloride and paracetic acid (NaClO/ $C_2H_4O_3$)) disinfections on palastic surface.

Biofilm cells of *S. typhimurium* were formed at different incubation time and temperature on the plastic surface used in this study. Therefore, considering the incubation temperature, the biofilm cells of *S. typhimurium* that were formed at a high incubation indicated an average sensitivity to all the disinfectant. Treatment conducted on biofilm cells that were incubated at 10 ^{o}C (refrigeration temperature) indicated high sensitivity to the treatments than the other temperatures used with a high percentage. Then followed by those biofilm cells that were formed on surfaces at 37 ^{o}C and the lowest sensitivity percentage were observed on the biofilm formed at 28 ^{o}C (room temperature) incubation. At this point, incubation time referred to the contact time used during incubation. Furthermore, this is a period through which bacterial cells replicates and form a biofilm on plastic surfaces. The biofilm cells formed on the pirates of the plastic wells increase continuously because as the biofilm cells replicate they become larger and more mature which leading them becoming a full community, therefore during the usual rinsing conducted at each interval, the cells will not be rinsed off. This was proven and reported by (Pui *et al*., 2011b). The sensitivity of all the four sets of disinfectant on biofilm cells of *S. typhimurium* was summarized in figure 7 respectively. The figure was indicating the total percentage killed biofilm cells of *S. typhimurium* following a 6 minutes exposure following disinfectants (H_2O_2, NaClO, PA and PA/NaOCl) on a food contact surface (plastic surface). The bars in figure 7 represented the total percentage killed biofilm cells of *S. typhimurium* by each disinfectant on the surfaces in this study. Hydrogen peroxide (H_2O_2) with 91% indicated the highest percentage of killed biofilm cells of *S. typhimurium*, then followed by the mixture of paracetic acid and sodium hyphochloride (NaOCl/PA) with 70% and paracetic acid with 67 % being the third disinfectant, then the least was sodium hyphocloride with 25 %, percentage killed biofilm cells of *S. typhimurium* at different incubation temperature and time. Biofilm formation activity of *S. typhimurium* on different food contact surfaces and food processing industries have a very serious implication because indirectly it enters the food processing areas either along with the vegetables, meats or even through people that are working around whom are already infected with this pathogen (*S. typhimurium*) may likely conveyed it, and spread it all over the environment which can be easily be contacted, simply because of its ability to form biofilm cells on a plastic surface and other surfaces like (glass, wood, stainless steel) that may be available in the environment. Many of these materials are used as immediate surfaces in different activities like conveying of processed and non-processed foods materials from one place to another, in some situations this surface are even the one used in the cooking of these foods. An important thing about biofilm is that, the issue of contact time of this pathogenic bacteria on a surfaces that enables it to formed a matured biofilm cells on surfaces, but depending on the durations and types of activities employed during processing of the food, some procedures may take different time and also on the other hand this bacteria used to form biofilm cells based on time intervals the more the time the higher the population. That is why we (mimic this process of time course in bacterial generations) considering this range of hours in this study and make our incubation time to follow the same pattern (24, 48 and 72) hours so that *S. typhimurium* cells will be able to form a matured biofilm. Because according to previous reports, the effects of many disinfectants on biofilms is depending on the age of the biofilm, duration or time of exposure of the biofilm cells by the disinfectant and the concentration of the disinfectant used for a particular treatment (Moretro *et al*., 2009). Perhaps the way and manners on how people used equipment together with the way they approached cleaning activities in their kitchen or food processing areas by addition of disinfectants may be another significant contribution on the rampant spreading of diseases. But the mistakes they usually made here is that, when the biofilm is formed on any surface around, may easily be the source of cross contamination possibly as a result of circulation of this food within the processing areas. Another suggestion may be the exposure time

used in this study, which was 6 minutes. May be the time used for the exposure to disinfectants treatment may be very short to kill all the biofilm cells of *S. typhimurium* formed on the surfaces. Therefore, if a longer period may be used, it may likely kill all the resisted biofilm cells. 6 minutes was chosen based on consideration of the types of disinfectant we used in this study. But in a different study also with different chemical disinfectant, *S. typhimurium* biofilm cells were exposed to 5 minutes treatment with chlorine and hypochloride disinfectants which inactivated the bacterial cells (Ramesh *et al*., 2002). A mixture of the two disinfectants (Paracetic acid and Sodium hypochloride) influences more sensitivity of *S. typhimurium* biofilm cells compared to their individual effect, before the mixture. The highest percentage of the killed biofilm cells reached up to 77 % and the lowest is 64 % on biofilm cells of *S. typhimurium* formed, this clearly indicated that, the mixture of the two disinfectants is more sensitive to biofilm cells of *S. typhimurium* than, the individual effect of the two disinfectant used before mixture. The effect indicated by Paracetic acid and sodium hypochloride in this study was also reflected in the previous report by Ramesh *et al*., (2002), where they reported a 5 minute exposure of the two disinfectant that inactivated the biofilm cells of *S. typhimurium* (Ramesh *et al*., 2002). According to the ranking made by the healthcare- associated infections (HAIs, 2012) during the process of surveillance for the infectious diseases, like blood stream infections, catherter-associated urinary tract infections and ventilator-associated pneumonia, they considered chemical disinfectants are the most significance defenses against (HAIs, 2012). Healthcare management analyzed and decided the best ten (10) best disinfectants that satisfied their procedures and experiments in order to fulfil their needs, they used the global Data profiled of the ten most important disinfections solution as indicated that; number 1 Formaldehyde, 2 Glutaraldehyde,3 Ortho-phthalaldehyde, 4 Hydrogen peroxide, 5 Peracetic acid, 6 Hydrogen peroxide/peracetic acid combination, 7 Sodium hypochlorite, 8 Iodophors, 9 Phenols, 10 Quaternary ammonium compounds (HAIs, 2012). Hydrogen peroxide is number four (4) best disinfectant, Paracetic acid is number three (3) best disinfectant and sodium hypochloride is number seven (7) best disinfectants according to the hierarchy. And this was also similar to the outcome of this study, Hydrogen peroxide indicated the highest percentage killed biofilm cells, hence is the number one (best) disinfectant, followed by the mixture of paracetic acid & sodium hypochloride as the second best disinfectant and sodium hypochloride was the least disinfectant used in this study. Hydrogen peroxide as a disinfectant solution containing about 7.8% hydrogen peroxide had been selected and approved by the USFDA, in order to be used for the high level process of sterilization and disinfection in healthcare settings (HAIs, 2012). In this study hydrogen peroxide (H_2O_2) with 91% indicated the highest percentage of killed biofilm cells of *S. typhimurium*, this explained how effective hydrogen peroxide is on the biofilm cells of *S. typhimurium* and this outcome reflected a report made that, hydrogen peroxide as a disinfectant solution contained about 7.5% hydrogen peroxide, had been selected and approved by the USFDA, for the process of high-level sterilization and disinfection in healthcare settings (HAIs, 2012). The second disinfectant that indicated an effective sensitivity of biofilm cells of *S. typhimurium* was a mixture of paracetic acid and sodium hypchloride (NaOCl/PA) with 70% sensitivity. Paracetic acid with 67 % being the third effective disinfectant, then the least was sodium hypochloride with 25 %, percentage killed biofilm cells of *S. typhimurium* at different incubation temperature and time. The result obtained in this study on the effect indicated by paracetic acid and sodium hypochloride was similar to report on a study conducted were a mixture of about nine commercial disinfectants for a comparison, sodium hypochloride had the lowest effect and paracetic acid indicated an intermediate effect on the biofilm cells of *S. typhimurium* (Moretro *et al*., 2009). On the other hand a very high percentage of the *S. typhimurium* cells indicated a very high sensitivity to the treatment of the four sets of disinfectant used in this study while a very small percentage resisted the action of these treatments. Nevertheless the outcome is excellent because sometime the biofilm cells that were formed on various surfaces developed a different mechanism of resistivity, like the production of some physiological changes in their metabolisms or sometime secretion of enzymes that can provide them with protection against actions of disinfectants and even destroying the composition of some antibiotics (Leriche *et al*., 2000).

Also today, many chemical manufacturing companies produced most of the disinfectants with a very low active composition which enhanced resistivity of the microbial cells (Leriche *et al*., 2000).

CONCLUSION

For a food contact surfaces particularly plastic surface, contamination can occur from different sources elsewhere, because in this study plastic surface had been assessed and confirmed to supported and formed biofilm with *S. tpyhimurium* cells, as a result of biofilm formation indicated to be a potent factor today that contributes to the frequent cross-contamination that lead to a persistent source of disease outbreaks. Therefore hygienic practice during handling, processing, distribution and storage of food on plastic surfaces or materials associated with the plastic surface is a significant issue to the public healthcare concern. This indicated that, the formation of biofilm by *S. typhimurium* on plastic surfaces varied with contact time. For the disinfection treatment of *S. typhimurium* cells and other related serovars on food associated utensils like plastic surface or in the hospital settings as an antibiotic, we suggested hydrogen peroxide, a mixture of sodium hypochloride and paracetic acid or paracetic acid separately to be used.

ACKNOWLEDGEMENTS

The financial support for the success of this study was shouldered to my humble government and UniSZA in particular. Furthermore, the extensive used by facilities concerning the progress of the study was extended to UniSZA.

REFERENCES

Antunes P, Réu C, Sousa JC, Peixe L, Pestana N. Incidence of Salmonella from poultry products and their susceptibility to antimicrobial agents. Int J Food Microbiol, 2003; 82 (2): 97-103. http://www.sciencedirect.com/science/article/pii/S0168160502002519 [Accessed 10 July 2015]

Bendinger B, Rijnaarts HHM, Altendorf K, and Zehnder AJB. Physicochemical cell surface and adhesive properties of coryneform bacteria related to the presence and chain-length of mycolic acids. Appl Environ Microbiol, 1993; 59 (11): 3973–3977. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC182562/ [Accessed 10 July 2015]

Center for Disease Control and Prevention. Surveillence for food borne disease outbreaks- United States, 2009-2010. Morbidity and Mortality Weekly Report, 2013; 62 (03):41-47. http://www.cdc.gov/mmwr/preview/mmwrhtml/mm6203a1.htm [Accessed 10 July 2015]

Chavant P, Gaillard-Martinie B, Talon R, Hébraud M, Bernardi T. A new device for rapid evaluation of biofilm formation potential by bacteria. J Microbiol Methods, 2007; 68 (3): 605-612. http://www.sciencedirect.com/science/article/pii/S0167701206003381 [Accessed 10 July 2015]

Chmielewski RAN, Frank JF. Biofilm formation and control in food processing facilities. Int J Food Sci Tech, 2003; 2 (1): 22-32. http://onlinelibrary.wiley.com/doi/10.1111/j.1541-4337.2003.tb00012.x/pdf [Accessed 10 July 2015]

Donlan RM, and Costerton WJ. Biofilms: Survival Mechanisms of Clinically Relevant Microorganisms. Clinical Microbiology Reviews, 2002; 15 (2): 167-193. http://cmr.asm.org/content/15/2/167.short [Accessed 10 July 2015]

Donlan RM. Biofilms: Microbial life on surfaces. Emerg Infect Dis, 2002; 8 (9): 881-890. http://wwwnc.cdc.gov/eid/article/8/9/02-0063_article [Accessed 10 July 2015]

Gilbert P, McBain AJ, and Rickard AH. Formation of microbial biofilm in hygienic situations: A problem of control. Int Biodeterior Biodegradation. 2003; 51 (4): 245-248. http://www.sciencedirect.com/science/article/pii/S096483050300043X [Accessed 10 July 2015]

Hall-Stoodley L and Stoodley P. Biofilm formation and dispersal and the transmission of human pathogens. Trends in Microbiol, 2005; 13(1):7-10. http://www.sciencedirect.com/science/article/pii/S0966842X04002586 [Accessed 10 July 2015]

Kostaki M, Chorianopoulos N, Braxou E, Nychas GJ, & Giaouris E. Differential biofilm formation and chemical disinfection resistance of sessile cells of Listeria monocytogenes strains under monospecies and dual-species (with Salmonella enterica) conditions. Appl Environ Microbiol, 2012; 78(8): 2586-2595. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3318796/

Leriche V, Sibille P, Carpentier B. Use of an enzyme-linked lectinsorbent assay to monitor the shift in polysaccharide composition in bacterial biofilms. Appl Environ Microbiol, 2000; 66(5):1851-1856. http://aem.asm.org/content/66/5/1851.full [Accessed 10 July 2015]

Mai T, and Conner D. Effect of temperature and growth media on the attachment of *Listeria monocytogenes*to stainless steel. Int J Food Microbiol, 2007; 120 (3): 282-286. http://www.sciencedirect.com/science/article/pii/S0168160507005107

Maki MD, Dennis G. Coming to grips with food borne infection-peanut butter, peppers, and Nationwide Salmonella Outbreaks. New Engl J Med, 2009; 360 (10):949-953. http://www.nejm.org/doi/full/10.1056/NEJMp0806575 [Accessed 10 July 2015]

Merritt JH, Kadouri DE and Toole GA. Growing and Analyzing Static Biofilms. Curr Protoc Microbiol, 2005; DOI: 10.1002/9780471729259. mc01b01s22 http://onlinelibrary.wiley.com/doi/10.1002/9780471729259.mc01b01s22/pdf

Møretrø T, Vestby LK, Nesse LL, Storheim SE, Kotlarz K, Langsrud S. Evaluation of efficiency of disinfectants against *Salmonella* from the feed industry. J Appl Microbiol, 2009; 106 (3): 1005-1012. http://onlinelibrary.wiley.com/doi/10.1111/j.1365-2672.2008.04067.x/epdf [Accessed 10 July 2015]

Oh S, Chen P, and Kang D. Biofilm formation by *Enterobacter sakazakii* grown in artificial broth and infant milk formula on plastic surface. Journal of Rapid Methods and Automation in Microbiology, 2007; 15 (4): 311-319. http://onlinelibrary. wiley.com/doi/10.1111/j.1745-4581.2007.00103.x/full [Accessed 10 July 2015]

Pui CF, Wong WC, Chai LC, Lee HY, Nillian E,Ghazali FM, Cheah YK, Nakaguchi Y, Nishibuchi M and Radu S. Simultaneous detection of *Salmonella* spp., *Salmonella* Typhi and *Salmonella typhimurium* in sliced fruits using multiplex PCR. Food Control, 2011a; 22 (2): 337-342. https://www.deepdyve.com/lp/elsevier/simultaneous-detection-of-salmonella-spp-salmonella-typhi-and-2h0sOwbjQf [Accessed 10 July 2015]

Pui CF, Wong WC, Chai LC, Lee HY, Tang JYH, Noorlis A, Farinazleen MG, Cheah YK and Son R. Biofilm formation by *Salmonella* Typhi and *Salmonella typhimurium* on plastic cutting board and its transfer to dragon fruit. International Food Research Journal, 2011b; 18: 31-38. http://www.mycite.my/en/article/articles-citing/article/11005 [Accessed10 July 2015]

Ramesh N, Joseph SW, Carr LE, Douglass LW and Wheaton FW. Evaluation of Chemical Disinfectants for the Elimination of *Salmonella* Biofilms from Poultry Transport Containers. Poultry Science, 2002; 81 (6): 904-910. http://ps.oxfordjournals.org/content/ 81/6/904.full.pdf [Accessed 10 July 2015]

Stepanović S, Ćirković I., Ranin L. and Vabić-Vlahović MS. Biofilm formation by *Salmonella* spp. and *Listeria monocytogenes*on plastic surface. Lett Appl Microbiol, 2004; 38 (5): 428-432. http://onlinelibrary.wiley.com/doi/10.1111/j.1472-765X.2004.01513.x/pdf [Accessed 10 July 2015]

Ukuku DO, and Fett WF. Relationship of cell surface charge and hydrophobicity to strength of attachment of bacteria to cantaloupe rind. J Food Prot, 2002; 65(7): 1093-1099. http://www.researchgate.net/publication/11262043 [Accessed 10 July 2015]

Van Houdt R and Michiels CW. Biofilm formation and the food industry, a focus on the bacterial outer surface. J Appl Microbiol, 2010; 109(4): 1117-1131. http://onlinelibrary.wiley.com/doi/10.1111/j.1365-2672.2010.04756.x/full [Accessed 10 July 2015]

Van Merode AE, van der Mei HC, Busscher HJ, & Krom BP. Influence of culture heterogeneity in cell surface charge on adhesion and biofilm formation by Enterococcus faecalis. J Bacteriol, 2006; 188 (7): 2421–2426. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1428413/ [Accessed 08 August 2015].

Kable, a trading division of Kable Intelligence Limited. Top ten disinfectants to control HAIs. 2012. http://www.hospitalmanagement.net/features/featureppc-disinfectants-hai-globaldata/ [Accessed 08 August 2015].

How to cite this article:

Sani Aliyu, Nor Iza A. Rahman, Anas Muazu, Umar Faruk Abdullahi, Rochman Naim, Umma Muhammad, Mainul Haque. Evaluation of Biofilm formation and Chemical Sensivity of *Salmonella typhimurium* on plastic surface. J App Pharm Sci, 2015; 5 (10): 118-125.