International Research Journal of Advanced Engineering and Science
ISSN (Online): 2455-9024

Impact of PVC and Stainless-Steel Materials on *Staphylococcus aureus* Biofilms Production and their Thermodeactivation as Affected by pH, Time and NaCl

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***Abstract*—** *Staphylococcus aureus is an opportunistic and ubiquitous pathogen capable of developing into a biofilm on work surfaces, processing surfaces or implants causing cross-contamination of food and humans. Heat treatment is the method that has shown effectiveness against the elimination of biofilms on work surfaces. However, the treatment is not always effective. The objective of this study was to evaluate the effect of some growth factors on the formation and thermo-deactivation of S. aureus biofilms on PVC and stainless-steel materials. S. aureus ATCC 13565 was firstly grown in nutrient broth at 37°C at different pH (5 – 7), NaCl (0,005-8%) and incubation duration (0-48h) following a CCD experimental design and their biofilm formation on stainless steel and PVC material assessed. A CCD design was also used to assess the inactivation at 60°C of biofilms formed on both materials after 24h of incubation with as variables the previous pH and NaCl conditions and the heat treatment duration (0-30mins). For all the tested conditions, Staphylococcus aureus biofilms formation was higher on PVC than on stainless steel. In contrast to NaCl, pH and incubation time factors showed a significant influence ($p<0.05$) on biofilm formation. Indeed, pH tending towards neutrality and long incubation time, lead to an increase in biofilm formation on the materials. Regarding thermal inactivation, the maximum deactivation was observed both on stainless steel and on PVC in condition with pH: 6; NaCl: 4% and time 15mins at a rate of 60% against 50 % respectively. Acidic growth conditions appeared to later be the most heat resistant. A quadratic effect of salt was also noticed with PVC. Roughness material such as PVC increases the formation of biofilms and limits heat treatment efficiency compared to stainless steel. Their use in food systems shall be limited.*

***Keywords*—** *Biofilms, environmental factors, PVC, stainless-steel, staphylococcus aureus, thermal inactivation.*

I. INTRODUCTION

Bacterial biofilms are communities of bacteria which are protected by a self-synthesized layer of complex polysaccharides, proteins, lipids and extracellular DNA, referred to as extracellular polymeric substance (EPS) (Flemming et *al*., 2017). They play a bioremediation role and are essentials in water treatment due to their ability to degrade pollutants carried by effluents (Von., 2007). However, they are also the source of many problems for food and medical industries. Indeed, the are quite often associated to materials bio-corrosion, food safety concern, alteration of the organoleptic qualities of food products and chronic wounds, nosocomial infections, antibiotic resistance (Abdallah, M et *al* 2010 & 2014; Gardete et *al*., 2014). They have been reported as responsible of a variety of infections in humans, 65% of which have been recorded in developed countries (Attinger, C et al., 2012; Romling, U et al., 2012). Among the harmful biofilm producing microorganisms, *Staphylococcus aureus* takes a very important place (Katharina Richter et *al*., 2017). It is an opportunistic, ubiquitous pathogen that prevalently colonizes PVC and stainless-steel working surfaces that are commonly found in the food and medical industries (Rodrigue LR et *al*., 2011; Souza et *al*., 2014). Highly resistant to various environmental conditions, most strains of *Staphylococcus aureus* have the capacity to cause severe infections when ingested through food or when they infect wounds and tissues to progress to severe food poisoning, fatal diseases such as pneumonia or necrotizing fasciitis (Hang Yang et *al*., 2017). Several means of control have been adopted to limit the harmful effects of biofilms. These include: cleaning-in-place techniques, biocides using chemical disinfectants for industry and antibiotic lock for medical staff (Oliveira C et *al*., 2012). However, these methods have limited effectiveness due to insufficient penetration of the products into the biofilm and the fact that most antimicrobials are designed to control bacteria in the free state and not in biofilm (DeBeer et *al*., 1994). Heat treatment when applicable is a safe physical method that has a much more positive image in the control of biofilm on work surfaces. Despite the proven effectiveness of this method, there is often a problem of resistance or variability in the effectiveness of the treatment due to the spatio-temporal heterogeneity of the biofilms (Jacques & tremblay, 2010). Several studies such as those of Jacques & tremblay (2010) have shown that the growth condition of microorganisms affects their thermo-resistance. On the other side surface conditioning, type of growth medium, temperature, pH,

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electrostatic and physical interactions between bacterial cells and the surface, cell-cell communication and signalling are all factors that can influence bacterial adhesion and subsequent biofilm development (Palmer et *al*., 2007). It could therefore be hypothesised a link between this biofilm condition formations and thermal resistance. Indeed, this raises the question of how material factors and environmental conditions influence the formation of biofilm and inactivation probability. With *S. aureus* as target microorganism, this study aimed at predicting heat inactivation efficiency of biofilm on the basis of the formation condition.

II. Materials and Methods

A. Materials

a) Biological material

The *Staphylococcus aureus* strain (ATCC 13565) used in this work was provided by the Microbiology Laboratory of the University of Bologna in Italy. It was sub-cultured thrice in nutrient broth before being used, each culture being done at 37°C for 24 hours.

b) Support for fixing biofilms

Polyvinyl chloride (PVC) and stainless-steel coupons, each 4 cm long and 1.5 cm wide, were used as a support for fixing the biofilms.

B. Methods

a). Experimental design

A Central composite design (CCD) was used to study the effect of variables: "pH, NaCl and incubation duration" and "pH, NaCl and heat treatment duration" on biofilm formation and thermal inactivation respectively. The levels used are reported in Tables I and II.

b). Evaluation of biofilm formation by S. aureus on PVC and stainless-steel materials in the tested conditions

Different media were prepared in jars according to the conditions of the CCD plan for a final volume of 130ml and sterilized. They consisted in Nutrient broth adjusted to the tested NaCl concentration and the correct pH. This media was inoculated with the strain for an initial concentration of 6 Log cell/ml. in each of them, were then introduced aseptically 3 stainless steel and 3 PVC sterile coupons and the media incubated at 37°C. the media volume in the jars was enough to totally cover the surface of the coupons. The quantity of biofilms formed on each coupon after the incubation period was evaluated on spectrophotometric basis (Djordjevic et *al*. 2002). In fact, once the cultures were taken out of the incubator, each coupon was washed 5 times with sterile distilled water, dried at room temperature for 45 min, immersed in 1% purple crystal for 45 min, rinsed with distilled water and introduced into 10 mL of 95% ethanol solution. The optical density of the obtained ethanolic solution was read at 595 nm using spectrophotometer with ethanol as blanc.

c). Evaluation of heat resistance of S. aureus biofilms to heat treatments formation under different conditions

The formation of the biofilms was firstly done at 37°C for 24h at pH and salt concentrations following the tested CCD experimental design (Table II). However, it is rather 10 coupons of each material that were included in the growth media. After incubation, the coupons were taken out of the jars, introduced aseptically into individual plastic bags containing 8 mL of sterile nutrient broth. The latter were then double-sealed and thermally treated for the defined duration (rf CCD), using a water bath previously set at the working temperature (60 °C). Once the processing time was reached, the individual sachets were cooled with cryogenic solution (-18°C) to immediately stop heat effect and incubated at 37°C for 24 h. The heat resistance could therefore be evaluated by counting the number of sachets that shows turbidity after this regrowth period over the 10 tested (1).

$$degree\ of\ heat\ resistance = \left(\frac{Number\ of\ turbid\ sachets}{10} * 100\right) \quad (1)$$

c). Statistical analysis of results

In order to assess the link between response and factors, a multiple regression was performed and factors and combinations of factors statistically significant at $P < 0,05$ were retained for the equation. This was done using Statistica 10 software from Statsoft. The probability of biofilm heat resistance (P) at the studied temperature was calculated using a logistic approach and based on the degree of resistance observed in each tested condition.

$$P = logit\ (p) = f(ph, NaCl, t) \quad (2)$$

$$logit\ (p) = \ln\left(\frac{p}{1-p}\right) \quad (3)$$

$$Pd = \frac{e^p}{e^p + 1} \quad (4)$$

$$Prb = 1 - Pd \quad (5)$$

$$Prb = 1 - \left(\frac{e^p}{e^p + 1}\right) \quad (6)$$

With

Prb: is the probability of resistance to heat treatment

p: the regression equation which is a function of the parameters studied

Pd: Probability of deactivation

III. Results

A. Quantification of the S. aureus Biofilms Formed on PVC and Stainless-Steel Materials under the Different Growing Conditions Tested

Table I presents the results obtained from the measurement of the quantity of biofilms, formed under the different tested conditions. Whatever the tested condition, the formation of biofilm was more important on PVC material than on stainless steel material. In both materials the lowest optical density was noted in conditions at pH=6; NaCl=4%; t=24h while the highest was obtained in condition 19 (pH=7; NaCl=8%; t=48h) for stainless steel and condition 8 (pH=6.5; NaCl=2%; t=12h) for PVC.

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TABLE I: OD values obtained under different biofilm formation conditions

Run	pH	NaCl (%)	t (h)	DO obtenues (moyenne ± écart type)	
				Inox	Pvc
01	5,5	2	12	0,056 ± 0,004	0,124 ± 0,016
02	5,5	6	36	0,056 ± 0,004	0,110 ± 0,034
03	6,5	2	36	0,062 ± 0,014	0,137 ± 0,024
04	6,5	6	12	0,054 ± 0,008	0,102 ± 0,012
05	6	4	24	0,043 ± 0,007	0,100 ± 0,018
06	5,5	2	36	0,059 ± 0,009	0,140 ± 0,010
07	5,5	6	12	0,054 ± 0,006	0,109 ± 0,010
08	6,5	2	12	0,073 ± 0,004	0,144 ± 0,024
09	6,5	6	36	0,068 ± 0,007	0,128 ± 0,004
10	6	4	24	0,043 ± 0,009	0,077 ± 0,021
11	5	4	24	0,052 ± 0,010	0,107 ± 0,016
12	7	4	24	0,058 ± 0,003	0,109 ± 0,012
13	6	0,005	24	0,052 ± 0,007	0,102 ± 0,037
14	6	8	24	0,069 ± 0,003	0,121 ± 0,023
15	6	4	0	0	0
16	6	4	48	0,067 ± 0,008	0,137 ± 0,023
17	6	4	24	0,053 ± 0,012	0,095 ± 0,015
18	7	0,005	48	0,071 ± 0,008	0,116 ± 0,006
19	7	8	48	0,076 ± 0,013	0,136 ± 0,017
20	5	0,005	0	0	0

t = incubation time; *PVC* = Polyvinyl Chloride

In order to assess the link between response and factors, a multiple regression was performed and factors and combination of factors statistically significant at $P < 0.05$ were retained for the equation hence the following equations for predicting biofilm production according to the growth condition on stainless steel and PVC materials obtained was as follows.

$$Qb = \exp(-7{,}60 + 0{,}66pH + 0{,}75\sqrt{t} - 0{,}1pH\sqrt{t} \quad Inox \quad (7)$$

$$Qb = \exp(-7{,}17 + 0{,}7pH + 0{,}93\sqrt{t} - 0{,}13pH\sqrt{t} \quad PVC \quad (8)$$

With *t* = incubation time;

Qb = Quantity of biofilm formed.

No significant effect of salt concentration was observed on stainless steel and PVC. Only pH and incubation time had a significant effect on biofilm formation. A positive linear effect of these two variables was noted when assuming a logarithm effect on the quantity of biofilms formed with time. In fact, the increase in incubation time and pH towards values close to neutral leads to an increase in the production of biofilms on both stainless steel (Fig. 1) and PVC (Fig. 2). A weak interaction between pH and incubation time was also noted on both materials. However, the weight of this was very small compared to the linear effect of these two variables with regard to the regression coefficients obtained.

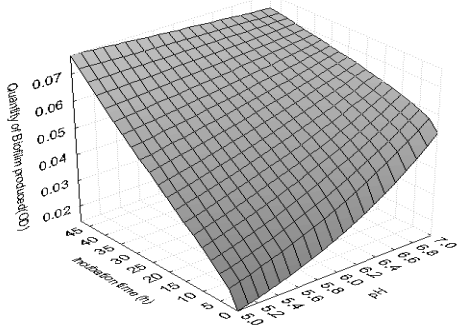

Fig. 1: Effect of pH and incubation time on biofilm formation by *S. aureus* on stainless steel material

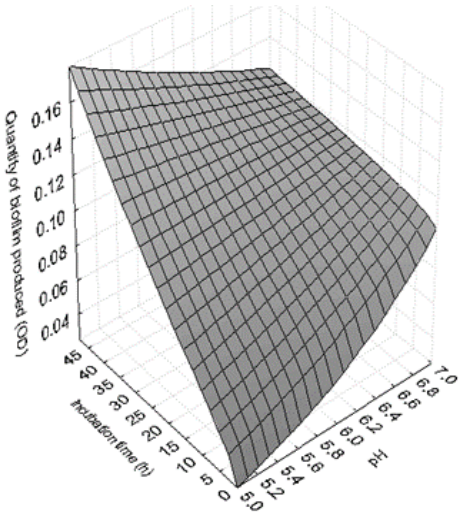

Fig. 2: Effect of pH and incubation time on biofilm formation by *S. aureus* on PVC material

B. Probability of Resistance of Biofilms to Heat Treatment at 60°C Depending on the Formation Conditions

Table II shows the different degrees of inactivation obtained after heat treatment of the biofilms formed on the coupons after 24 h incubation. Inactivation was significantly higher on stainless steel material than on PVC material, except under certain conditions. The lowest degree of resistance on both materials was obtained in condition 17 (pH 6; NaCl 4; treatment time 15 min) with an inactivation level of 50% and 60% on PVC and stainless steel respectively. Globally, biofilms formed at the most acidic pH were more resistant to the heat treatment.

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International Research Journal of Advanced Engineering and Science
ISSN (Online): 2455-9024

TABLE II: Degrees of biofilm resistance to deactivation according to the conditions of the CCD plan

Run	pH	NaCl (%)	Tps (mins)	Degré de désactivation (%)	
				Inox	Pvc
01	5,5	2	7	0	0
02	5,5	6	22	0	0
03	6,5	2	22	0	0
04	6,5	6	7	0	0
05	6	4	15	0	0
06	5,5	2	22	0	0
07	5,5	6	7	0	0
08	6,5	2	7	0	0
09	6,5	6	22	30	0
10	6	4	15	10	0
11	5	4	15	20	30
12	7	4	15	50	40
13	6	0,005	15	30	30
14	6	8	15	10	40
15	6	4	0	0	0
16	6	4	30	30	40
17	6	4	15	60	50
18	7	0,005	30	30	20
19	7	8	30	50	40
20	5	0,005	0	0	0

t = incubation time; *PVC* = Polyvinyl Chloride.

In order to assess the link between response and factors, a multiple regression analysis was performed and factors that had a statistically significant impact ($P < 0.05$) on the probability of biofilm resistance, resulting in the following equations:

$$Prb = 1 - \frac{\exp(-8{,}48 + 0{,}54\sqrt{dt} + 1{,}38\ log pH^2)}{1 + \exp(-8{,}48 + 0{,}54\sqrt{dt} + 1{,}38 log\ pH^2)} \quad Stainless - steel \quad (9)$$

$$Prb = 1 - \frac{\exp(-4{,}85 + 0{,}42\sqrt{t} + 0{,}34 logpHNaCl + 0{,}18 log\ NaCl^2)}{1 + \exp(-4{,}85 + 0{,}42\sqrt{t} + 0{,}34 logpHNaCl + 0{,}18\ log NaCl^2)} \quad PVC \quad (10)$$

Where *t* = Treatment time;

Prb = Probability of biofilm resistance.

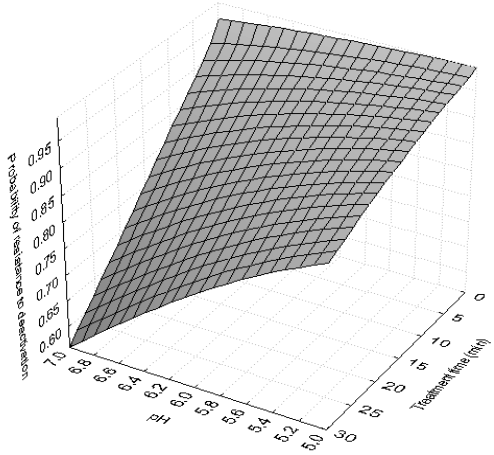
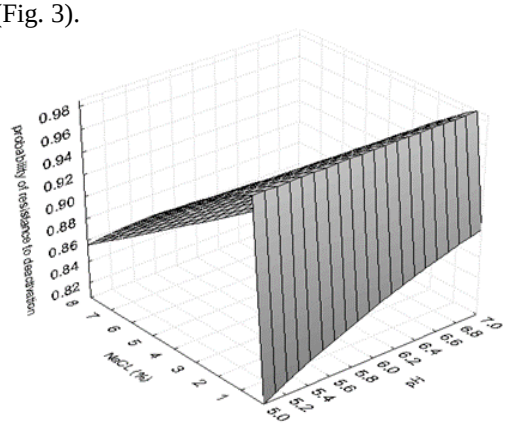
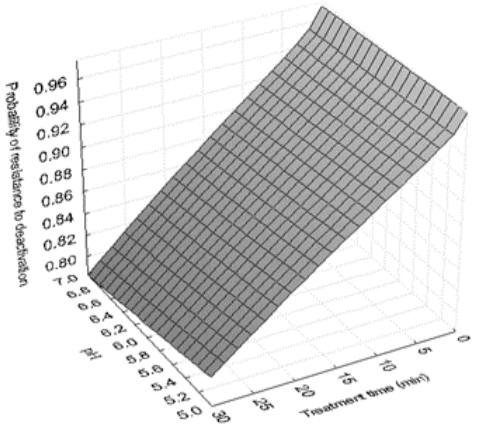


Fig. 3: Effect of pH and treatment time on the probability of biofilm resistance at 60°C on stainless steel

The probability of resistance was significantly influenced by the 03 factors tested on PVC material; whereas on stainless steel, only pH and treatment time seemed to have a significant effect on this response. A linear effect of incubation time and a quadratic effect of pH were noted on the regression function (in the case of stainless steel). The increase in treatment time reduced the probability of biofilm resistance, but biofilms formed at the most acidic pH tested were significantly more resistant than those formed at pH close to neutral on stainless steel (Fig. 3).



(a)



(b)

Fig. 4: Effect of NaCl and pH on the probability of biofilm resistance at 60 °C at a fixed treatment time value (7 min) (A) and of pH and treatment time at a fixed value of NaCl (4%) (B) on PVC material.

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On PVC material a significant quadratic effect of NaCl was noted. Indeed, by keeping the treatment time fixed at 7 min for example, the probability of biofilm resistance at 60°C increases for NaCl values ranging from 0 to 4% and decreases beyond this range (Fig. 4A). At the same time, a significant linear effect of treatment time was also observed. The increase in time decreased the probability of resistance (Fig. 4B).

IV. Discussion

Substrate and environmental characteristics are known to influence cell adhesion during biofilm formation (Jeronimo et *al*., 2012). Several studies have reported the ability of bacteria to form biofilms on materials that are commonly used in the food and medical sectors such as: stainless steel, glass, rubber, polycarbonate, polyurethane, polystyrene, polypropylene, titanium, aluminum and ceramics (Simões et *al*., 2010; Vazquez-Sanchez et *al*., 2013; Hamadi et *al*., 2014). In this study, *S. aureus* exhibited an ability to form biofilms on both PVC and stainless steel with a preference for PVC. In fact, in contrast to stainless steel, PVC has a stiffer structure with asperities that gives it a larger adhesion surface, which is therefore suitable for biofilms formation (Izabela Biedroń1 et *al*., 2017). The analysis of some growth parameters has shown that, pH and incubation duration, taken individually or in combination had a significantly positive correlation with the formation of the biofilms on the materials used. The NaCl at the tested concentrations (0,005-8%) did not have a significant influence on that biofilm formation. This result is in line with the work of Jeronimo et *al*, (2012) who noted that by growing *S. aureus* in BHI enriched with 5% NaCl no increase in adhesion capacity and biofilm formation was observed. On the other hand, Moretro et *al*. (2003) observed that a 2% NaCl concentration resulted in an increase in the adhesion and biofilm-forming capacity of *S. aureus* on polypropylene and stainless steel. This difference in results may be due to the difference in the culture medium used, nutrient broth in ours case and BHI in theirs.

Increasing the pH to values close to neutral lead to an increase in the formation of biofilms. This is due not only to the pH range used, which is close to the optimal pH for growth of *S. aureus* (pH=7) and which is favorable for biofilm formation, but also to a low electrostatic repulsion between the cells and the support, which increases as the pH rises (Meinders et al., 1994). This result is close to that obtained by Tarek et *al*, (2009) who showed that *S. aureus* biofilm formation at pH = 5 was low and opposite to Hamadi et *al*, (2010) who showed that *S. aureus* adhere strongly at pH values ranging from 4 to 6. Similarly, the significantly positive effect of incubation time on biofilm formation can be explained by a fairly long contact time between the cells and the support favorable to cell adhesion.

Evaluation of the effect of growth factors on the thermodeactivation process employed has shown that there is more deactivation on stainless steel than on PVC. In other words, the probability of resistance on PVC is greater on the latter than on stainless steel. This observation can be explained by a higher heat transfer on stainless steel material compared to PVC.

With regard to the influence of pH, the formation of biofilms at extreme pH levels subjects them to stress, resulting in a specific type of biofilm when they manage to overcome it. Indeed, growth in an acidic environment gives biofilms an increased resistance to heat (Fernandez et *al*., 2009). This would be explained by the fact that at pH lower than the optimal pH of the microorganisms, the cell tends to enrich its membrane with saturated fatty acids. This gives it greater rigidity and greater thermal resistance (Fernandez et *al*., 2009) due to a decrease in the unsaturated fatty acids / saturated fatty acids ratio, which leads to a reduction in membrane fluidity (Wang et *al*., 2005). This explains the greater resistance to heat treatment observed in at acidic growth pH conditions. However, the fact that the variable pH taken individually does not influence the resistance of biofilms on PVC shows that the nature of the materials has an impact on thermodeactivation; biofilms formed on stainless steel are significantly more sensitive than those formed on PVC (Simmonds et *al*., 2003).

The increase in treatment time leads to a decrease in the probability of biofilm resistance at 60 °C on the materials used. This observation has confirmed the knowledge already acquired regarding the influence of treatment time on the resistance of a biofilm. Indeed, the microbial thermo-deactivation is a function of the time-temperature couple and a more intense treatment contributes to weaken or even denature the surface proteins of the cells. This destabilizes the membrane and reduces the resistance capacity of the biofilm.

The NaCl concentration also significantly influenced the thermo-deactivation. Indeed, the relationship with thermoresistance has always been of the quadratic type, i.e. increases resistance at low concentrations and limits it after a certain threshold. The adhesion of salt on PVC material has been higher than that observed on stainless steel and therefore its influence on the biofilms formed would be greater on PVC.

V. Conclusion

In conclusion, this work has shown that *Staphylococcus aureus* forms biofilms preferentially on PVC material compared to stainless steel and that this formation is increased for pH values close to neutral and for relatively long periods of incubation time. The probability of resistance of the *S. aureus* biofilm is higher on PVC material and is reduced at pH values tending towards neutrality and high treatment times.

The strict respect of cleaning process is therefore an imperative especially in food industries with that PVC material instalments to avoid contamination risk.

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