DISINFECTANT TESTING FOR BIOFILM ELIMINATION USING MODIFIED CAPACITY TEST

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ABSTRACT

Two disinfectants, chlorine solution and the mixture of peracetic acid and hydrogen peroxide solution (PAHP), which widely used in the food industry were examined for their efficiency to eliminate the biofilms compare to the suspended cells using modified capacity test. The tested concentrations of chlorine (200 ppm) and PAHP (0.2% and 0.4%) were effective against the cell suspension of the two test organisms, *Staphylococcus aureus* and *Pseudomomas fluorescens* (at least 8 log reduction). No viable colony was observed even when the disinfectants were diluted into half strength of the tested concentration. Whereas the biofilm of the same test organisms gave unsatisfactory results, in particular with the used concentration of PAHP. Furthermore milk showed the greater interfering capacity against the PAHP activity than the chlorine activity when testing with biofilms. Therefore to ensure the full efficiency of disinfection regime in the food industry, the modified capacity test should be applied with the biofilms rather than testing with the planktonic cells.

KEYWORDS: Biofilm, *Pseudomonas fluorescens*, *Staphylococcus aureus*, capacity test, disinfectants, peroxyacetic acid, hydrogen peroxide, chlorine

1. INTRODUCTION

Food related microorganisms whether pathogen or spoilage are become more aware in the food industry over the past two decades [1]. Cleaning and disinfection are required regime for the food processing plants [2]. Therefore disinfection testing for the efficiency of minimizing the contaminated bacteria is essential. The efficacy of disinfectants before being commercialized can be tested in different techniques, basically there are two major methods. One is determination of reduction number of viable test microorganisms (i.e. the hard-carrier test), the other is examination of the recommend-used concentration when it being exposed with microorganisms and organic matter (i.e. the capacity test) [3]. The capacity test, thus, reflects the suitable concentration that is able to inactivate the test strains down to an acceptable level. Likewise guideline standards for testing the efficiency of the disinfectant (i.e British & European standard (BS-EN-1040 [4], UNE-EN-1276 [5]) only provide the methods for testing with suspended cells. Brinez *et al.* [6] and Holah *et al.* [7] reported of employing the European standard in testing of bactericidal activity of several disinfectants, i.e. quaternary ammonium compounds, sodium hypochlorite and PAHP. They found that the used concentration of such disinfectants achieved the decimal reduction of 5 log order of suspended test bacteria.

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However microorganisms have a nature to attach to the surface in order to gain advantage in their survival [8]. Such attached cells which known as biofilms were reported from laboratory trials to be 10 – 100 times more resistant to treatment with many biocides than the cell suspension of the same organisms [9]. In practice it might not be appropriate to used suspended cells to be tested with the disinfectants when those finally will be applied to eliminate the organisms that attach to the floor and the surface of equipments. Thus this research aimed to develop the method to evaluate the efficiency of disinfectants that suitable for eliminating the biofilms by modifying the capacity test which normally being tested with cell suspension. The test organisms used in this study were *Pseudomonas fluorescens* and *Staphylococcus aureus*. The former is one of the bacteria generally found in natural environment as well as in many areas of food processing factory and is one of the food spoilage organisms which can grow at refrigeration temperature [10]. The latter is considered as foodborne pathogen and hygiene indicator of the food handler [11]. The ultimate purpose was also to conduct a potential approach in testing of manufacturer's recommended concentration of disinfectants prior to use in the industry.

2. MATERIALS AND METHODS

2.1 Bacterial strains

Dairy farm isolates of two test bacteria, *Pseudomonas fluorescens* (PH) and *Staphylococcus aureus* (SE), were grown and maintained on nutrient agar (NA) at 4°C. The cultures were transferred three consecutive days and grown overnight in NA slant at 37 °C prior to testing.

2.2 Cell suspension preparation

Overnight cultures in NA were suspended in phosphate buffered saline (PBS; pH 7.2) to an OD_{600} of 0.5 in order to obtain a viable cell number of $10^8 – 10^9$ CFU/ml.

2.3 Surface material & cell attachment

The stainless steel coupons (type 304, food grade, 1.5 cm by 2.6 cm; Unitech Science Co. Ltd., Bangkok, Thailand) were washed with dish washing detergent (Sunlight, Unilever Thai Holding Ltd.) and soaked in distilled water before dry sterilization at 180 °C for 3 h. To produce the pre-existing biofilms, the coupons were immersed vertically in 20 ml of the cell suspension in 50 ml polypropylene centrifuge tube and incubated at room temperature for 90 min (the time suitable for the highest number of attachment as previously reported by Vatanyoopaisarn [12]). Coupons were removed and rinsed four times with 10 ml of PBS. Additionally each rinsing the coupons were tapped gently by holding vertically on sterile tissue paper to remove the excess liquid droplets.

2.4 Disinfectant Testing

The disinfectant substances used in this study were commercially available and widely used in food industries and animal farms in Thailand and in many other countries. Those were 200 ppm chlorine solution (prepared from dehydrated calcium hypochlorite) and the mixture of peracetic acid and hydrogen peroxide or PAHP (Oxonia Active, Ecolab); this disinfectant solution was prepared according to the manufacturer' s recommendation into the concentrations of 0.2% and 0.4% (v/v).

UHT fresh milk (Thai Denmark) was used as interfering substance in order to see the influence of organic matter on the effect of the disinfectants and to imitate the practical conditions of the food industry. Cell suspension in PBS at an OD_{600} of 0.5 was ten-fold diluted with the UHT fresh milk.

The efficiency of the disinfectants to kill the suspension cell was examined regarding the capacity test described in Harrigan [3]. Briefly, 1 ml of cell suspension (either in PBS or UHT milk) was placed to 6 ml of the disinfectant in the steriled test tube. At 10-min intervals 1 ml of the bacterial suspension was added and mixed gently. A sample was withdrawn 8 min after each addition, five single drops of 0.02 ml were placed on the surface of pre-dried plate count agar (PCA). This procedure was continued for 1 h, with six additions of the test organism until the concentration of disinfectant being approximately half diluted. The plates were incubate at 37°C for up to 48 h. The highest number of additions that gives fewer than 5 colonies from 5 drops accounted as the end point of the test. The best efficiency of the disinfectant concentration regarded as satisfactory if three or more increments can be added before a positive culture was obtained (> 5 colonies/ 0.1 ml/ plate).

The disinfectant testing for biofilm elimination was performed using modified capacity test, 6 coupons of pre-existing biofilms as described above were immersed into 6 ml of the disinfectant in the centrifuge tube. At 10-min intervals, 1 ml of PBS or interfering substance was added up to six times. A coupon was removed after 8 min of exposure times and rinsed four times as mentioned previously. The stainless steel coupon then was placed into 10 ml of neutralizer (0.01 M Sodium thiosulfate, pH 7) [13] followed by the enumeration procedure.

2.5 Recovery of attached cells for enumeration

The rinsed coupons were placed into 5 ml of maximum recovery diluent (MRD) (OXOID Unipath Ltd.) in a new universal tube and the cells were detached by vortexing continuously for 2 min as previously used in Vatanyoopaisarn [14]. The survival cells was determined by spotting five single drop of 0.02 ml of the released cells on PCA and incubated at 37°C for up to 48 h before observing the colonies. The satisfactory result obtained from the disinfectant concentration that can withstand at least three additions of PBS or UHT milk and gave fewer than 5 colonies per plate (or < 5 colonies/ 0.1 ml).

The initial number of cell suspension and the initial number of the attached cells were also plate counted. The experiments were repeated twice.

3. RESULTS AND DISCUSSION

3.1 Capacity test of chlorine solution.

The bactericidal effect of 200 ppm chlorine solution of suspended cells and biofilm of *S. aureus* (SE) and *P. fluorescens* (PH) is shown in Tables 1-2. The bacterial cell suspensions were killed in all tests, eventhough the addition of 1 ml of cell suspension was carried out up to 6 times. The initial cell numbers of *P. fluorescens* and *S. aureus* were 8.9 $\pm$ 0.09 log CFU/ml and 8.6 $\pm$ 0.04 log CFU/ml, respectively. This reflects that at least 8 log of suspended cells of the two test organisms were killed at the concentrations used. Furthermore the UHT milk showed no interfering capacity when testing with suspended cells. The similar concentration of chlorine solution was applied to the biofilm on stainless steel of the same test bacteria with the initial attached cell number of 4.5 $\pm$ 0.03 log CFU/cm^2 (*P. fluorescens*) and 4.2 $\pm$ 0.04 log CFU/cm^2 (*S. aureus*). The in-use concentration gave satisfactory results since more than three increments of additions were able to perform before the colony counts exceeded five colonies per plate [3]. Although it should be noted that addition of UHT milk was likely to interfere the disinfectant efficiency when the milk was 5 times added into *S. aureus* biofilm and 6 times added into *P. fluorescens* biofilm (Table 2).

The chlorine-based compounds have been approved to use for treatment of water and in the food industry for more than five decades, and the concentrations widely applied for sanitizing in the food industry are between 100 – 200 ppm [15]. Although it was evident that mutagenic chemical by-products can be derived from chlorine [16], the low cost and easy accessible of the chemical make it still being popularly used.

Table 1 Bactericidal testing of 200 ppm chlorine solution to impair suspended cells by capacity test

Number of additions at 10-min interval	Number of colonies / 0.1 ml			
	S. aureus		*P. fluorescens*	
	PBS suspension	Milk suspension	PBS suspension	Milk suspension
1	0	0	0	0
2	0	0	0	0
3	0	0	0	0
4	0	0	0	0
5	0	0	0	0
6	0	0	0	0

Table 2 Bactericidal testing of 200 ppm chlorine solution to impair biofilms by modified capacity test

Number of additions at 10-min interval	Number of colonies / 0.1 ml			
	S. aureus		*P. fluorescens*	
	PBS addition	Milk addition	PBS addition	Milk addition
1	0	0	0	0
2	0	0	0	0
3	0	0	0	0
4	0	0	0	0
5	0	25	0	0
6	0	515	0	25

Data represent the mean of colony count of 4 replicates.

3.2 Capacity test of peracetic acid and hydrogen peroxide mixture (PAHP).

The biocide effect of 0.2% and 0.4% PAHP against cell suspensions is shown in Table 3. The results were similar to the test of chlorine solution which indicated complete elimination up to half-diluted of the used concentration. The initial cell numbers of *S. aureus* and *P. fluorescens* were 8.8 $\pm$ 0.11 log CFU/ml and 9.1 $\pm$ 0.06 log CFU/ml, respectively. Thus approximate reduction of at least 8 log was obtained in cell suspension in the presence and absence of organic matter. This work partly agree with the study of Brinnez *et al.* [6] which showed that the PAHP varied from 0.05 – 0.4% were able to inactivate the suspended cells of *S. aureus*, *Escherichia coli* and *Listeria monocytogenes* more than 5.0 log decimal reduction in the presence and absence of milk. .

The results of the modified capacity test conducted with the biofilms are shown in Table 4. The PAHP merely passed the test when the highest concentration (0.4%) was exploited. It was also clear that the higher concentration (0.4%) was more effective to reduce *S. aureus* and *P. fluorescens* than the lower concentration (0.2%). Moreover the UHT milk showed interfering capacity in the two test bacteria as the second addition of milk led to the detection of uncountable grown colonies on the plate. However the initial attached cells of *S. aureus* and *P. fluorescens* in this experiment were 6.2 $\pm$ 0.03 log CFU/cm^2 and 7.0 $\pm$ 0.02 log CFU/cm^2, respectively , which higher than the biofilms number used in treatment of chlorine.

Thus this could be estimated that 5 log reduction was achieved in an absence of organic substance, whereas in the presence of milk, almost full concentration of disinfectants was required. The PAHP, a mixture of peroxyacetic acid (5.8%) and hydrogen peroxide (27.5 %) [17], is commonly used for CIP (cleaning-in-place) in many food processing factories worldwide [7] and the safe recommended concentration is limited to 0.4% [18]. Thus it is very important that microbial contamination occurring in food processing area must be minimized and the concentration used must be sufficient to prohibit the occurrence of biofilm to the level lower than 3 log CFU/cm^2 in order to achieve disinfection procedure.

According to the European standard (EN 1040), 5 log units reduction of vegetative cells must be achieved in order to consider that a certain disinfectant has a satisfactory germicidal activity, where the guideline of standard proposed of testing with the cell suspension [4]. From this work it clearly indicated that the chlorine and PAHP used concentrations has higher efficiency than the requirement when the cell suspension being tested. However when testing with the biofilms of the same strains, it showed that disinfectants only passed the minimum requirement when the maximum concentration were used. As in practice these disinfectants will be employed to destroy the organisms contaminate on the floor or working surfaces, the biofilms are then more suitable for pre-testing the activity of disinfectants at the manufacturer's recommended concentration. Additionally, because the capacity test method only requires the results of the absence colony of the known initial cell number which also make less tedious works on serially diluted cell of plate counting, therefore modified capacity test as shown in this study could be a choice of application.

Table 3 Bactericidal effect of 0.2% and 0.4% of PAHP to impair suspended cells

Number of additions at 10-min interval	Number of colonies / 0.1 ml							
	S. aureus				*P. fluorescens*			
	PBS suspension		Milk suspension		PBS suspension		Milk suspension	
	0.2 % PAHP	0.4 % PAHP	0.2 % PAHP	0.4 % PAHP	0.2 % PAHP	0.4 % PAHP	0.2 % PAHP	0.4 % PAHP
1	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0

Table 4 Bactericidal testing of 0.2% and 0.4% of PAHP to reduce biofilm by modified capacity test

Number of additions at 10-min interval	Number of colonies / 0.1 ml							
	S. aureus				*P. fluorescens*			
	PBS addition		Milk addition		PBS addition		Milk addition	
	0.2 % PAHP	0.4 % PAHP	0.2 % PAHP	0.4 % PAHP	0.2 % PAHP	0.4 % PAHP	0.2 % PAHP	0.4 % PAHP
1	0	1	0	0	0	0	0	1.5
2	2	0	0	1.5	0	1	TN	1
3	38	0.5	TN	5.0	0	0.5	TN	TN
4	61	0.5	TN	TN	78	1	TN	TN
5	TN	0.5	TN	TN	TN	1	TN	TN
6	TN	1	TN	TN	TN	1	TN	TN

Data represented the average of colony count, n= 4; TN surviving cells were too numerous to count.

4. CONCLUSIONS

The recommended use concentration of chlorine solution and PAHP were effective against the suspended cells of *S. aureus* and *P. fluorescens* up to 8 log reduction, but only achieved approximately 3 – 4 log reduction when the biofilm of the same strains were exploited. The modified capacity test was a possible disinfectant testing to conduct with the biofilm prior to the application at its normal in-use concentration.

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