LWT - Food Science and Technology 56 (2014) 58–61

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

Peracetic acid disinfectant efficacy against *Pseudomonas aeruginosa* biofilms on polystyrene surfaces and comparison between methods to measure it

M.C. Martín-Espada [a], A. D'ors [b], M.C. Bartolomé [c], M. Pereira [b], S. Sánchez-Fortún [b,*]

[a] Dpto. Sanidad Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, Av. Puerta de Hierro s/n, 28040 Madrid, Spain
[b] Dpto. Toxicología y Farmacología, Facultad de Veterinaria, Universidad Complutense de Madrid, Av. Puerta de Hierro s/n, 28040 Madrid, Spain
[c] Facultad de Quimicofarmacobiología, Universidad Michoacana de San Nicolás de Hidalgo, Calle Santiago Tapia, n° 43, 58000 Morelia, Michoacán, Mexico

ARTICLE INFO

Article history:
Received 18 June 2013
Received in revised form
28 October 2013
Accepted 9 November 2013

Keywords:
Peracetic acid
Biofilm
Pseudomonas aeruginosa
Polystyrene surfaces
Cleaning and disinfection

ABSTRACT

In food industry, biofilms are a source of recalcitrant contaminations, being possible sources of public health problems. The aims of this study were evaluate the disinfectant efficacy of peracetic acid against *Pseudomonas aeruginosa* biofilms grown on polystyrene surfaces, determining the best method to measuring its disinfecting efficacy. PERAsafe® has been used as peracetic acid donor into medium. Direct colony-forming units (CFUs) counts together with two assays based in optical density measurement have been tested. Starting from bacterial suspensions ($\sim 10^5$), the $\log_{10}$ values obtained were 4.34 ± 0.20, 2.60 ± 0.77 and 0.00 for 0.4%, 0.8% and 1.61%, PERAsafe® concentrations, respectively, showing that this compound achieved the 100% killing efficacy against *P. aeruginosa* biofilms. Comparative study among the selected tests shows similar results between CFUs counts and spectrophotometry, but there are significant differences between direct counting and McFarland test. At concentration of 1.61%, the correlation factors were 0.8998 and 0.7338 when comparing CFU direct counting with microplate absorbance measurements and McFarland turbidity tests, respectively. These results show that measurement on microplates is an effective tool to evaluate the effectiveness of PERAsafe® against *P. aeruginosa* biofilms, being able to effectively replace the CFUs count in routine tests to determine the sterilization levels on surfaces.

1. Introduction

The presence of viable microorganisms on a surface presents a potential risk. These microorganisms may be actively multiplying and colonizing the surface, or may be merely surviving, retaining viability but being unable to multiply due to adverse environmental conditions (Veran, 2002). Most cells survive by adhering to a surface after which they hide under a layer of polysaccharides. In time this becomes a biofilm where different kinds of microorganisms can live together (Poulsen, 1999).

One organism of particular concern within the healthcare environment is the multi-drug resistant *Pseudomonas aeruginosa*. This microorganism is a ubiquitous environmental bacterium that has become a major opportunistic pathogen among immunocompromised patients in intensive care units or patients in neonatal or burns units, and is the principal cause of chronic infection, leading to lung damage, in cystic fibrosis sufferers (Rossolini & Mantengoli, 2005). Bacteria have been isolated from a variety of surfaces (Brooks, Walczak, Hameed, & Coonan, 2002; Lyczak, Cannon, & Pier, 2000) growing in the natural environment attached to surfaces in biofilms (Epstein, Hochbaum, Philseok & Aizenberg, 2011).

Biofilms have been described in many systems since Van Leeuwenhoek examined the *animalcules* in the plaque on his own teeth in the seventeenth century, but the general theory of biofilm predominance was not promulgated until 1978 (Costerton, Geesey, & Cheng, 1978). Data on which this theory is predicated came mostly from natural aquatic ecosystems, in which direct microscopic observations and direct quantitative recovery techniques showed unequivocally that more than 99.9% of the bacteria grow in biofilms on a wide variety of surfaces.

Microbial biofilms are notorious for their high level of resistance towards biocidal treatments, and the mechanisms associated with such resistance are complex (Marsh, 2005). These mechanisms involve not only the reaction-diffusion limitation of biocide access to the underlying cells (Gilbert, Das, Jones, & Allison, 2001), but also

* Corresponding author. Tel.: +34 13943841.
E-mail address: fortun@ucm.es (S. Sánchez-Fortún).

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http://dx.doi.org/10.1016/j.lwt.2013.11.013

the expression of spatially heterogeneous, less susceptible phenotypes, caused either by growth as a biofilm *per se* (Al-Jailawi, Ameen, & Al-Jeboori, 2013) or through the expression of high cell density (Lenz, Williamson, Pitts, Stewart, & Franklin, 2008), or starvation (Hunt, Werner, Huang, Hamilton, & Stewart, 2004).

Peracetic acid is an ideal antimicrobial agent due to its high oxidizing potential. It is highly effective against a broad range of microorganisms. Because of its effectiveness against bacteria, fungi, and viruses, peracetic acid is used as a disinfectant in the food and medical industries (Lewis, 1993; Wallace, Agee, & Demicco, 1995).

In this study, the efficacy of PERAsafe®, which liberates peracetate ions equivalent to 0.26% peracetic acid into the medium, was tested on biofilms of the multi-drug-resistant *P. aeruginosa* grown on polystyrene surface. The disinfectant activity on *P. aeruginosa* biofilms was tested in three different analytical methods to determine which of them provided a higher sensitive level.

2. Material and methods

2.1. Chemical

The disinfecting efficacy of peracetic acid on biofilms has been evaluated. The disinfectant has been applied in the tests as PERAsafe®, commercial formulation supplied by DuPont Co. (DuPont Company, Suffolk, UK). PERAsafe® stock solutions were prepared immediately before use and filtered through a 0.22-μm sterilizing filter. All other reagents and chemicals were purchased from Sigma (Sigma–Aldrich Chemie GmbH, Switzerland).

2.2. Bacterial strain and preparation of inoculum

P. aeruginosa (NCTC 122924) type strain was obtained from the Spanish Type Culture Collection (CECT; University of Valencia, Spain). The microorganisms were grown on Trypcase-Soja agar (TSA; bioMérieux® S.A., Marcy l'Etoile, France) 90 mm-plates, at 37 °C. One full loop was transferred to 250 ml of Brain Heart Infusion broth (BHI-D; bioMérieux® S.A., Marcy l'Etoile, France) and incubated at 37 °C for 16 h, until the population levels were 10^7 CFU ml^{-1}.

2.3. Biofilm formation

Starting from an overnight liquid culture, dilutions containing approximately 100 CFUs ml^{-1} were made. For each biofilm experiment, 20 wells of a round-bottomed polystyrene 24-well microtiter plate (IWAKI, Tokyo, Japan) were inoculated with 100 μl of these dilutions, and 4 control wells were filled with sterile medium. Following 4 h of adhesion, the supernatant (containing non-adhered cells) was removed from each well and plates were rinsed using 100 μl physiological saline solution (PS). Subsequently, 100 μl of fresh medium was added to each well and the plates were further incubated for 24 h. After 4 h adhesion and 24 h biofilm formation, the supernatant was again removed and the wells were rinsed with 100 μl of PS.

The dynamics of adhesion and biofilm formation was controlled by standard biomass assays in polystyrene 24-well microtiter plates using crystal violet, according to the methodology proposed by Christensen et al. (1985).

2.4. Biofilm assay

Solutions containing selected PERAsafe® concentrations (0.4%, 0.8% and 1.61%, equivalent to 1.04×10^{-3}%, 2.08×10^{-3}% and 4.186×10^{-3}% peracetic acid into the medium, respectively) were prepared and appropriate volume was added to microtitre plate wells individually. The total volume of each well was made up to 200 μl with BHI-D broth. The wells without disinfectant compound were used as controls, and the plates were incubated at 37 °C for 15 min under stationary conditions. At the end of the contact time, 200 μl of the appropriate neutralizer solution (DNP-F, bioMérieux® S.A., France) was added in order to neutralize any traces of disinfectant may have remained in the wells. The neutralizer was allowed on for 20 min to permit cell recovery. At least eight replicates were used for each sample, and each experiment was carried out at least four times.

In order to recover the surviving biofilm bacteria, after exposure biofilms were rinsed twice in PS and then they were disrupted into this by sonication by mean a water table sonicator Sonicor SC-52 (Best Chemical Co., China) for 15 min at 40 W. Thus, the samples are prepared for the implementation of the three methodologies proposed in this study.

2.5. Plates count

Viable counts were made by spreading 0.2 ml aliquots of appropriate dilutions, made in sterile saline, onto Trypcase-Soja agar (TSA; bioMérieux® S.A., Marcy l'Etoile, France) 90 mm-plates. They were incubated at 35 °C, and the colonies counted at 24-, 48-, and 72-h. The values were obtained as Colony Forming Units per cm^2 (CFUs), and the results were expressed as logarithmic values and as percentages respect to control.

2.6. Absorbance measurement in plate reader

Aliquots (0.1 ml) were placed, in quadruplicate, into the wells of Nunc® MicroWell™ 96 well polystyrene plates (Sigma–Aldrich Chemie GmbH, Switzerland). They were placed within an incubated (35 °C, moderate shaking) microtitre plate reader (Thermo Scientific Multiskan FC, Waltham, MA, USA), and absorbances (492 nm) were monitored at 24-, 48-, and 72-h. Initial absorbance readings for the filled plates were subtracted from the subsequent data set, and the results were expressed as percentages respect to control.

2.7. McFarland turbidity test

The same methodology, described for the determination of bacterial growth by measuring absorbance, has been applied to carry out McFarland test. Samples have been analyzed in densitometer (Densimat, bioMérieux® S.A., 69280 Marcy l'Etoile, Francia), and turbidities (550 nm) were monitored at the same time intervals. The values were obtained as McFarland units, and the results were expressed as percentages respect to control.

2.8. Statistical analysis

The log reduction is expressed as log (No/N), where No and N are initial and post- treatment microbial counts, respectively. Values are expressed as colony-forming units per cm^2 (CFU). The results were calculated as averages and standard deviations from eight experiments ($n = 8$). The estimation of population values from the sample data was performed using One-way Analysis of Variance (ANOVA), following of Bonferroni's Multiple Comparison Test. Values were considered significant at $p < 0.05$. The coefficient of determination (r^2) was calculated from the Pearson correlation coefficient (r). Statistical analysis was developed using the software package GraphPad Prism v5.0 (Graph-Pad Software Inc., USA).