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HOT WATER SANITIZATION AND ITS IMPACT ON MICROBIAL BIOFILM CAUSING BACTERIA *PSEUDOMONAS AERUGINOSA* AND *PSEUDOMONAS PUTIDA*

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Abstract – Biofilms are the challenge in maintenance of pharmaceutical water system. Generation of purified water or demineralized water and maintaining quality attributes are the key requirements as this water used for processing of medicinal products like tablets, capsules, syrups, nasal sprays, injectables etc. Before allowing for purification, it is manufacturer's responsibility to ensure the microbial load control in the source water to get good quality product water and to avoid biofilm formation in waterlines. Current research involves the study of an effectiveness of Hot water sanitization in control of Biofilm formation bacteria *Pseudomonas aeruginosa*. The aim of this study was to examine the effect of Hot water on bacterial growth by challenging known concentration of Biofilm causing bacteria *Pseudomonas aeruginosa*. The study conducted by selecting different temperatures like 50°C, 70 °C and 90 °C for holding the sample for different time points. Biofilm formation of *Pseudomonas aeruginosa* and other species like *Pseudomonas putida* is common issue in water systems, by using of two biofilm strains, sanitization effectiveness of hot water were examined.

INTRODUCTION

Hot water sanitization is common method used to sanitize the pharmaceutical water systems. (Sihorkar and Vyas, 2001; Beczner, 2001) Biofilms are matrices of microorganisms embedded in their own microbial-originated extracellular polymeric substances (EPSs) attached to a solid surface (Kisko *et al* 2011). Biofilms are a concern in a broad range of areas, causing slime problems, reducing heat transfer efficiency in pipelines of water system, heat exchangers and condensers, great hygienic and financial concerns in Pharmaceutical and other industrial applications (Sandle, 2015). Biofilms are particularly problematic in pharmaceutical industry (Armon *et al*., 1997). Bacteria associated biofilms are difficult to kill and remove from the inner surfaces of pharmaceutical pipelines (Tim Sandle 2017). The removal of a mature biofilm most often requires extensive mechanical action, such as scrubbing or scraping in conjunction with the use of cleaning and sanitizing agents (Simoes *et al*., 2006). Chemicals must be rinsed off from surfaces, leaving no toxic residues. By considering the limitations of sanitization by using chemical agents, Proposal of Hot water sanitation is an ultimate solution as it provides the green strategy for biofilm control (Tim Sandle, 2015). It has some advantages over chemicals: no chemical residues, no corrosion, and it can penetrate to hard-to-reach areas. The purpose of this study was to evaluate the removing and inactivating Biofilm removal of Pseudomonas strains using hot water sanitation on stainless steel surface (Kipnis *et al*., 2006).

Gram negative organisms are most common in water samples, especially *Pseudomonas aeruginosa* is a common contaminant (Kipnis *et al*., 2006) in most of the pharmaceutical water systems and this produces biofilms. Infections with *P. aeruginosa* can be acquired from community settings (hot tubs, Jacuzzis, swimming pools), but occur mainly in healthcare settings, especially in critical care units and following procedures that involve physical breaches in host defenses, such as surgical incisions and the use of invasive devices Jefferies, Cooper, Yam, and Clar *et al*., (2012) Kerr and Snelling, (2009). Populations at risk include neonates, patients with deep neutropenia, severely burned patients, patients

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with invasive devices (e.g., vascular and urinary catheters, endotracheal tube, ventilator), and patients who have underlying pulmonary disease such as bronchiectasis and cystic fibrosis Jefferies *et al*., (2012) Kerr and Snelling, (2009) Leclerc, Schwartzbrod and Dei Cas, (2002). *Pseudomonas aeruginosa* can cause a variety of infections, including pneumonia, bacteremia, urosepsis, and wound infections Berthelot *et al.,* (2001) de Victorica and Galvan, (2001) Leclerc *et al.* (2002). *Pseudomonas aeruginosa* is a bacterium widely recovered from the environment that is capable of colonizing a number of wet and moist sites in plants and soils and a wide variety of aquatic environments Hardalo and Edberg, (1997).

The steps involved in biofilm formation are (Figure 1):

1. Individual cells populate the surface (initial attachment)
2. Irreversible attachment
3. Extrapolymeric substances are produced, and attachment becomes irreversible
4. Biofilm architecture develops and matures

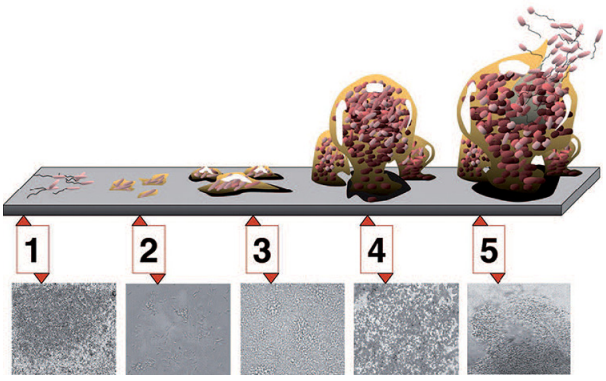


Fig. 1.

5. Single cells (or clumps of cells) are released from the biofilm over time

MATERIALS AND METHODS

Culture Media Preparation: All the media and chemicals used for present study were of Himedia laboratories, India. The required media were prepared and sterilized at 121 °C for 25 minutes (Lechevallier *et al*.,(1980). Soyabean casein digest agar, Normal saline used for cultures enumeration and R2A agar media used for testing of samples. Medias are prepared by using dehydrated media bottles (Massa *et al.,* 1998). Calculated the required media quantity and number of containers as per manufacturer recommendation. After weighing the required amount, transferred to the container which is filled with approximately 1/4th volume of the required quantity of Purified water with the help of measuring cylinder. Reconstituted the media as per the Manufacturer's instructions mentioned on the container label.

Mixed the bottle to dissolve completely and make up to final volume with purified water (Reasoner *et al.,* 1984). The pH of the media's are verified and found within the specified pH range of manufacturer. After pH verification, media dispensed in to 500 ml screw cap bottles containing 400 ml of media. Media loaded in autoclave and operated for sterilization for about 25 minutes. After sterilization also pH of the medium verified and found satisfactory.

Microbial Cultures preparation: Standard Microbial culture *Pseudomonas aeruginosa* NCTC 12924 (Fierer *et al*. 1967) selected for study. *Pseudomonas aeruginosa* (NCTC 12924) procured from a standard culture collection centre used for study. The other organism Pseudomonas putida selected for study is isolate of water sample. The organisms were serially diluted and plated by following pour plate technique on Soya bean casein digest agar and incubated at 48 hours at 32.5°C. Once after incubation, results reading performed and the desired dilutions are stored at 2-8°C till usage (Stephen Edberg *et al*., 1988).

Purified water sample collection and sterilization: Approximately 10litres of Purified water samples are collected from the purified water generation system in a stainless container and transported to laboratory. Total 50 bottles (250 ml) are prepared for study, added 100 ml of water in each bottle and sterilized in a steam sterilizer at 121 °C for 25 minutes.

Inoculum density: Cultures containing the inoculum concentration of 10^6 cells /ml.

Sample preparation and inoculation Procedure

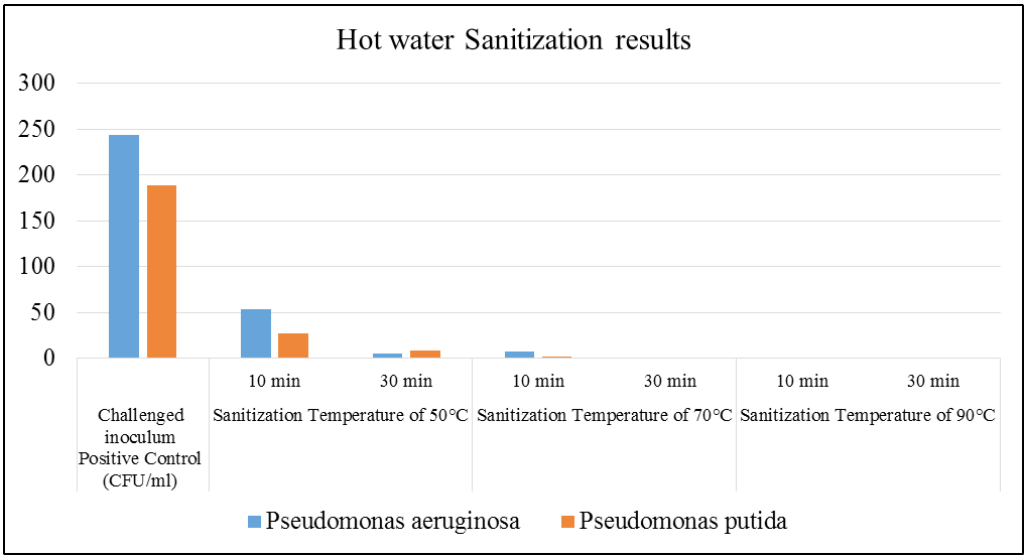
Two sets of preparations performed; each preparation contains 9 bottles.

- Total 9 bottles containing sterile purified water used for study. All the bottles are labelled with required details. First bottle served as negative control.
- Four bottles are selected for study of Pseudomonas aeruginosa, each bottle inoculated with 1 ml of $2.7x10^4$ culture suspension. sterile containers selected for study the

Results of Pseudomonas aeruginosa:

Organism name	Challenged inoculum Positive Control (CFU/ml)	Sanitization Temperature of 50°C		Sanitization Temperature of 70°C		Sanitization Temperature of 90°C	
		10 min	30 min	10 min	30 min	10 min	30 min
Pseudomonas aeruginosa	243	54	5	8	0	0	0
Pseudomonas putida	189	27	9	2	0	0	0

Negative control No growth



- Second set of four bottles are inoculated with the culture *Pseudomonas putida* culture suspension 1 ml contains $1.8x10^4$ cells / ml.
- Out of 4 bottles, 1 bottle for bottle for positive control and remaining 3 bottles for testing at different temperatures of each organism.
- The above procedure repeated for second set of 9 bottles.

Hot water sanitization & treatment:

Total 3 different temperatures are selected for study which are 50°C, 70°C and 90°C (Erica B *et al* 2018). From the above preparations two bottles of each culture, a total of 4 bottles collected and transferred to the steam pot which is previously maintaining at 50°C and allowed for 10 minutes contact time removed 2 bottles and allowed for room temperature. Remaining 2 bottles were allowed for 30 minutes contact time and taken out from steam pot and allowed for room temperature.

The above exercise performed for remaining 2 temperature such as 70°C and 90°C. (Brown and Hunter, 1977; Wrangstadh *et al.*, 1986; Kim and Frank 1994; Ryu *et al.*, 2004).

After heat treatment, all the exposed bottles along with controls are transferred to laminar air flow.

Total 9 bottles of each organism along with controls are segregated.

Pseudomonas aeruginosa: Pipetted 1 ml of test sample and transferred to the each of Petri plates in duplicates from each bottle. Added approximately 20-25ml of molten R2A agar media which was previously maintaining at 45°C. After addition of media, swirled the media plates clock and anti-clockwise to mix the sample homogeneously and allowed for solidification. After solidification, the plates are incubated in an inverted position at 32.5°C for 5 days.

Pseudomonas putida: Pipetted 1 ml of test sample and transferred to the each of Petri plates in duplicates from each bottle. Added approximately 20-25ml of molten R2A agar media which was previously maintaining at 45°C. After addition of media, swirled the media plates clock and anti-clockwise to mix the sample homogeneously and allowed for solidification. After solidification, the

plates are incubated in an inverted position at 32.5°C for 5 days.

RESULTS AND DISCUSSION

Pseudomonas family is the major predominant isolate in water systems. Ensuring the quality of water without Gram negative organisms are the toughest challenge in water generation and distribution systems. Two different *Pseudomon*as species are selected as standard organisms in this study. The challenged organisms *Pseudomonas aeruginosa* and *Pseudomonas putida* are having high capability to produce biofilms in water systems. Basis to that both organisms are selected for testing to establish control measures of these types of organisms. Biofilm formation is potentially increase ratio based on incubation time. The samples tested at 50 °C, 70 °C and 90 °C with two different contact times as 10 and 30 minutes.

The temperature range of 70 °C-90 °C shown very good control at 30 minutes contact time. Temperature of 90 °C shown no recovery at 10 minutes contact time. This study reveals that a frequent Hot water sanitization of water distribution system eliminates the biofilm formation to ensure the quality of water.

Sandle (2015) found that 2.5 x 102 CFU/ml of *B. Cepacia and 3.3 x 103 CFU of P. Fluorescens gave a detectable level of endotoxin with the LAL test (around 0.06 EU/ml)*

The second most populous isolate from WFI was *Burkholderia cepacia, or, more precisely, members of the Burkholderia cepacia complex (BCC), of which there are 18 different species*

The third most common isolated with WFI is *Flavimonas oryzihabitans, which was formerly classified as a Pseudomonad. The bacterium is closely related to Pseudomonas putida.*

Trautmann *et al. 2015 did not obtain acceptable long-term* results by using chlorination and filtration to eliminate the strains of *P. aeruginosa from every water tap.*

Methylotrophic bacteria have been found in several drinking water-related ecosystems (McCoy and VanBriesen, 2012; Douterelo *et al.,* 2014b; Liu *et al.,* 2014b)

CONCLUSION

Hot water sanitization is an effective method for sanitization of Purified water systems in pharmaceutical industries. Our results showed that the hot water sanitization reduced the number of Pseudomonas biofilm cells. Increase of contact time and the temperature shown significant drift on killing the bacterial cells. Frequent sanitization of pharmaceutical water system by using hot water is a good solution to eradicate biofilms in water systems. Hot water temperature ranges from 70°C to 90°C is shown positive results in controlling biofilms.

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