ISSN 0974-3618 (Print)
0974-360X (Online)

www.rjptonline.org

RESEARCH ARTICLE

Evaluation of Disinfectant action on Biofilm Bacteria

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ABSTRACT:
The control of potentially pathogenic bacteria in aquatic environments is of paramount importance. In the present study the effect of three disinfectants(concentration versus time) – phenol, formaldehyde, and Domex on bacteria in multispecies biofilm (MSB), single species biofilm of *E.coli*, *Enterococcus* spp., *klebsiella* spp., *Pseudomonas* spp.and *Staphylococcus aureus* and stable multispecies biofilm with *E.coli*, *Enterococcus* spp., *klebsiella* spp., *Pseudomonas* spp. and *Staphylococcus aureus* as indicators was determined by viable count method. There is a correlation between concentration of disinfectant and time.Initially with higher concentrations being more effective at 10 min. to 30 min. contact but at longer contact time of 60 min. to 120 min. the rate of killing is independent of concentration. Domex was found to be the most effective disinfectant especially against potentially pathogenic bacteria in biofilms. *Enterococcus* spp. was most susceptible to chlorine - at a concentration of 1 mg/l; while *E. coli* was most resistant requiring 30 min. to control in SSB and60 min. to control *E.coli* in multispecies biofilm. Similarly *Staphylococcus aureus* demonstrated highest resistance to phenol; *Klebsialla* spp. showed highest resistance to sodium hydroxide while the bacteria in single species biofilm (SSB) exhibited higher resistance to formaldehyde.

KEYWORDS: Disinfectants, Biofilm, multispecies biofilm, single species biofilm, *E.coli*, *Enterococcus* spp., *klebsiella* spp., *Pseudomonas* spp. ,*Staphylococcus aureus*, Chlorine.

1. INTRODUCTION:
Biofilms are one of the most complex associations of organisms. This initially involves bacteria followed by algae, fungi, protozoans and ciliates in biofilms that are more than 20 days old. The biofilm bacteria which colonize the surface of any substratum immersed in water sense the surface and undergoes change in its gene regulation pattern. This genetic shift results in an oligotropic bacterium that has the ability to form copious amounts of extracellular polymeric substances,EPS[1]. The EPS matrix is composed of polysaccharides, proteins and DNA[2].The altered state of biofilm organisms allows it to demonstrate varied response to disinfectant treatment as compared to planktonic forms[3]. The biofilm organisms recognize sense and respond to autoinducers via quorum sensing. This allows the microbial community to respond as a whole to any external stimulus[4]. The response of biofilm to action of disinfectants depends on the type of organism involved in biofilm formation[5] and also size of the biofilm matrix. The present study aims to identify the effective disinfectant for the control of biofilms.

MATERIALS AND METHODS:
Development of Biofilm:
The multispecies biofilm (MSB) was developed on sterile round bottomed polystyrene microtitre (ELISA) plate s with 200µl of tapwater. The plates were sealed and incubated at room temperature (30C) for 10 days. The resultant viable bacterial population was enumerated by first scrapping the well with sterile surgical blade. The entire contents were then aspirated with micropipette and aspirate suspended in 1 Ml of phosphate-buffered saline[6] (PBS; pH 7.2,). Each well was further rinsed four times with 200µl of PBS each time to ensure complete removal of biofilm. The contents were then centrifuged at 3000rpm (R-8C; REMI

Received on 10.04.2017 Modified on 24.05.2017
Accepted on 20.06.2017
Research J. Pharm. and Tech. 2018; 11(3): 910-912.
DOI: 10.5958/0974-360X.2018.00168.3

Centrifuge) for 10 Min. The resulting pellet was suspended in 100µl of PBS. The suspended cells were spread plated on Trypticase Soy Agar (TSA, Hi-Media, India) and incubated at 35C for two days[7].Decimal dilution and plating was performed wherever necessary.

The bacterial cultures – *E.coli, Klebsiella* spp., *Pseudomonas* spp., *Staphylococcus aureus*, and *Enterococcus faecalis*; were obtained as preserved stock from the department of Microbiology. The stock was checked for purity by plating on differential or selective media - MacConkey agarfor *E.coli, Klebsiella* spp., Pseudomonas agar for *Pseudomonas spp.*,Mannitol Salt Agar for*Staphylococcus aureus*and Enterococcus Agar for *Enterococci*(all media from Hi-Media, India). Four well isolated colonies from each plate was inoculated in 5 Ml of Trypticase Soy Broth respectively and incubated at 35C for 18H. The cells (~10^8cells perMl) were pelleted by centrifuging at 3000rpm for 10 Min. The pelleted cells were suspended by gentle aspiration in 200µl of PBS (pH 7.2). The cycle of centrifugation and washing with PBS was repeated three more times. Finally the pellet was suspended in 200µl of sterile tap water which was added to sterile microtitre wells, covered and incubated at 30C for 7 days to develop a stable single species biofilm(SSB). The initial number of viable cells was determined by standard plate count.

The MSB with indicator organism was developed by replacing 100 µl of tapwater from stable 10 days old MSB in microtitre well with 100 µl of respective indicator bacteria(obtained as described previously) – *E.coli, Klebsiella spp., Pseudomonas spp., Staphylococcus aureus* and *Enterococcus faecalis*; and further incubated at 30 C for four days. The sample from the MSB with indicator bacteria was plated on respective differential or selective media to ensure attachment of indicator organism. Samples from stable MSB were plated on indicator media to verify the absence of indicator bacteria in MSB. All results were calculated and presented as mean of three replicates ± Standard Deviation.

Evaluation of Disinfectant:

Phenol, formaldehyde (30%, AR grade) and Domex (commercially available brand) were serially diluted with filter sterilized distilled water to obtain dilutions of 1:4, 1:10 and 1:20. The dilutions once prepared were used within ten minutes. Disinfectants were added to MSB, SSB and MSB with indicator by replacing 100 µl of tap water from stable MSB/ SSB/ MSB with indicator in microtitre well with 100 µl of relevant disinfectant dilution. This gave a final dilution of 1:2, 1:5 and 1:10. The disinfectant was allowed to act for 10 Min., 20 Min., 30 Min. and 60 Min. Viable counts of organisms at the predetermined time intervals were obtained by spread plating on TSA for multispecies biofilm bacteria and multispecies biofilm bacteria with indicator and the numbers of indicator bacteria - *E. coli/ Klebsiella spp./ Pseudomonas spp./ Staphylococcus aureus/ Enterococcus faecalis* by plating on respective selective or differential media. All tests were performed as triplicates and biofilms without addition of disinfectant were used as controls. The contact time was optimized based on previous studies (data not shown).

RESULTS AND DISCUSSION:

The effect of Domex on biofilm bacteria are tabulated in fig.1; formaldehyde in fig.2 and phenol in fig. 3 revealed that Domex is the most efficient disinfectant among the three chemicals used in the study. This may be due to the fact that Domex contains sodium hypochlorite as a disinfectant plus a detergent, hydrotrope, deposition aid and pH regulator that maximizes its efficiency against biofilms. However, even Domex did not completely eliminate all biofilm bacteria and this agrees with the previous studies[8,9]. The effect of addition of indicator organism - *E.coli, Enterococcus* spp., *klebsiella* spp., *Pseudomonas* spp.and *Staphylococcus aureus* on resistance of biofilm bacteria followed a similar pattern of resistance as stated above – the higher numbers being due to the increase in the number of days for the biofilm formation. The effect may be partially due to penetration of chemicals into the biofilm[10] or due to amount and type of disinfectant as presented in table 1 which is agreement with the previous studies.[11, 12]

The cocci especially *Enterococcus* spp. demonstrated the maximum amount of resistance to the disinfectants followed by *Staphylococcus aureus*. *Klebsiella* spp. showed increased resistance to disinfectants while *Pseudomonas* spp. revealed least resistance Table 2. This is in agreement with the studies[13,14,15] carried out that stated that resistance may be due to a combination of factors – persisters, effective efflux pumps and quorum sensors.

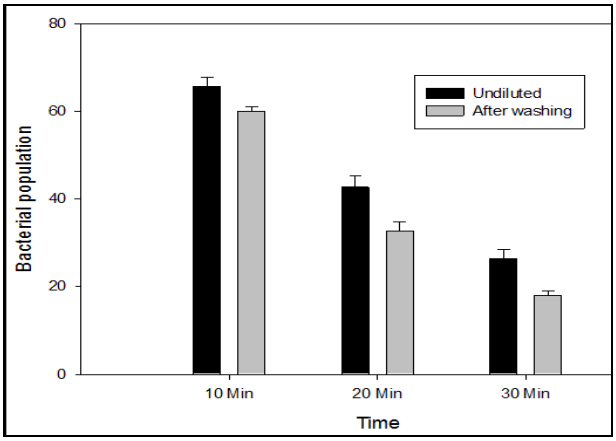

Fig. 1: Effect of Domex on multispecies biofilm bacteria

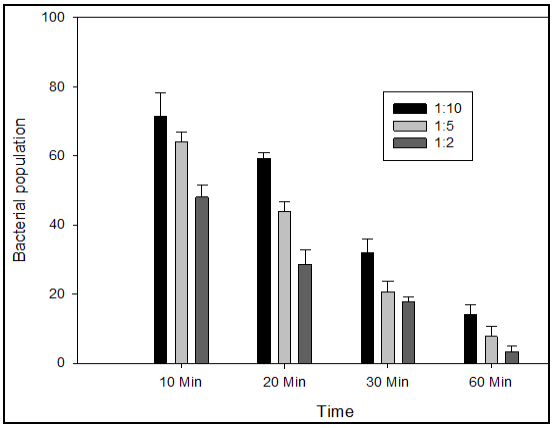

Fig. 2: Effect of formaldehyde on multispecies biofilm bacteria

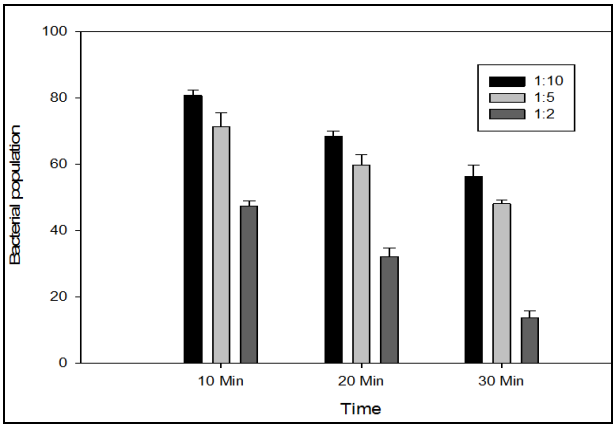

Fig. 3: Effect of phenol on Multispecies biofilm bacteria.

Table 1: Showing the effect of disinfectants as highest numbers of multispecies biofilm bacteria with indicator organism presented as concentration vs. time. [a] – MSB + *Klebsiella* spp. ; [b] – MSB + *Staphylococcus aureus.* All other data are for *Enterococcus* spp.SD – Standard deviation; Conc. – concentration used; NA – Not Applicable (not part of study design).

	Domex		**Formaldehyde**			**Phenol**		
	Undiluted	After washing	Conc. - 1:10	Conc. - 1:5	Conc. - 1:2	Conc. - 1:10	Conc. - 1:5	Conc. - 1:2
	Mean± SD	Mean± SD	Mean± SD	Mean± SD	Mean± SD	Mean± SD	Mean± SD	Mean± SD
10 Min	78±2.6	77.6±3.2	78.6±2.5	72±1	54.3±2.5	83.3±1.5[a]	79.6±2.1[b]	56.6±1.5[b]
20 Min	57±1	55.3±2.5	68.6±2.1	48.3±2.5	32.3±2.1	74.7±4[a]	68.3±1.5[b]	36.6±3.1[b]
30 Min	38±3.6	32±1	39.3±1.5	27.6±1.5	23±2	62±2.6[a]	61.3±1.5[b]	20.7±2.1[b]
60 Min	NA	NA	22.3±1.5	11±1	6±1	NA	NA	NA

Table 2: Showing the effect of disinfectants as highest numbers of indicator bacteria (*Enterococcus* spp.) attached multispecies biofilm - presented as concentration vs. time. SD – Standard deviation; Conc. – concentration used; NA – Not Applicable (not part of study design).

	Domex		**Formaldehyde**				**Phenol**	
	Undiluted	After washing	Conc. - 1:10	Conc. - 1:5	Conc. - 1:2	Conc. - 1:10	Conc. - 1:5	Conc. - 1:2
	Mean± SD	Mean± SD	Mean± SD	Mean± SD	Mean± SD	Mean± SD	Mean± SD	Mean± SD
10 Min	4±1	1.3±0.6	29±1	22.6±2.1	13.6±1.5	34.3±2.5	22.3±1.5	20.6±1.5
20 Min	0	0	20.3±1.5	7.6±1.5	32.3±2.1	28.7±1.5	12.3±2.1	13.6±1.5
30 Min	0	0	10±1	1.3±0.5	23±2	21.7±0.6	11.6±1.5	4.7±1.5
60 Min	NA	NA	5±1	0	0	NA	NA	NA

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