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Efficacy of Various Biocides Against Biofilm Bacteria Isolated from Cooling Waters of a Petrochemical Industry

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Cooling water is essential in many industries, including power generation, chemical manufacture and petroleum refineries. Microbial fouling, the formation of biofilms in cooling water systems can lead to reduced heat transfer in condensers and heat exchangers. In order to prevent biofilms from creating these problems, a number of biocides are being used. In this study, the minimal inhibition concentrations (MIC) against 18 biofilm bacteria (8 g-negative and 10 g-positive), isolated at different times from cooling waters of a petrochemical industry, with increasing amounts (4-64 ppm) of 10-different biocides were investigated. The experiments were carried out at pH 7.9 and at 30 ºC. Henkel P3 Ferrocid 8583 and Concorde chem Hydrobio 620 exhibited the greatest antimicrobial effect against isolated biofilm bacteria. In addition, the reduction of the numbers of viable microorganisms count in the samples taking from cooling solution by selected biocides is determined. Interestingly, the results of this study clearly demonstrate that the most effective biocides against isolated bacteria did not have the same level when they were tried with cooling solution.

Key Words: Biocide, Biofilm, Cooling water, Extracellular polysaccharide.

INTRODUCTION

Cooling water is essential in many industries, including power generation, chemical manufacture and petroleum refineries[1]. Microorganisms such as algae, bacteria can enter a cooling water system through the incoming water or through the air itself if a cooling tower is present.

Surface water used for cooling industrial processes contains many organic and inorganic substances as well as microorganisms. Surfaces that come in contact with this water will be directly conditioned by adsorption of the organic materials and bacteria. Mobile waterborne bacteria migrate to surfaces and attach by excretion of exopolysaccharides (EPS), forming gel-like matrices in which the bacteria are enclosed. Eventually areas may join together and form a continuous biofilm. A few operational problems in cooling water systems occur due to biofilm development.

These are increased corrosion, decreased efficiency of heat transfer, increased risk of macro-fouling and increase of health risks[2,3]. Therefore, the importance of

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an effective disinfection of cooling water is emphasized. The application of preventive or protective should also be made taking into account the biofilm. Consequently, cooling water is cleaner and biofouling of the pipes is minimized[4-6]. However, biocides due to their toxic nature can represent an ecological hazard. The obvious way to protect the environment is to avoid chemical treatment altogether, using physical technologies to reduce the impact of biofilms on efficient cooling water application[1]. In addition, certain bacteria could adopt and grow in the presence of biocides, which could lead to these bacteria becoming the dominant species[2]. It is essential that biocide which will be used for cooling water must be well selected.

The aim of this study is to determine effectiveness of various biocides at different concentrations against biofilm bacteria isolated from a cooling water system and at the reduction of the numbers of viable microorganisms count in the sample taking from cooling solution of a petrochemical industry.

EXPERIMENTAL

Isolation of the most exocellular polysaccharide producing bacteria isolated from cooling solution and thin biofilm layer of the cooling water system: To select the most exocellular polysaccharide (EPS) producing bacteria, samples taking from cooling solution and thin biofilm layers of cooling tower of a petrochemical industry (Izmir, Turkey) were passed aseptically through tenfold dilutions of Ringers solution to yield final concentrations of 10^{-4} and 10^{-5}. Spread plates were prepared from each dilution (0.1 mL) on nutrient agar (pH 7.9) and incubated at 30 ºC for 3 days. A total of 18 isolates that are mucoid colonies were purified by repeated isolation and subculturing on same medium at 30 ºC.

These isolates were picked from each plate into Erlenmeyer flasks (250 mL) containing exocellular polysaccharide medium (100 mL) and grown at 30 ºC with gyratory shaking (200 rev min^{-1}) for 5 days. The contents of each flask were then decanted in to 3 volumes of propan-2-ol, shaken vigorously and held at 4 ºC for 4 h. Exocellular polysaccharide producing organisms were identified by the appearance of strand-like mats of precipitated polysaccharide. The most EPS producing organisms were selected for this study[7-9].

Identification of isolates: The isolated bacteria were identified on the basis of the following features *i.e.*, colony morphology, colonial pigmentation, cell morphology, gram-staining reaction, oxidase positivity. Isolates were further characterized biochemically using the API 20E and 20NE kits (Analytical Profile Index, bioMérieux, Marcy l'Etoile, France).

Determination of effectiveness of various biocides against the isolated biofilm bacteria: Isolates were inoculated on 50 mL Triptic Soy Broth medium in 200 mL-flasks and grown at 30 ºC for 24 h. The cells harvested by centrifugation at 5000 rpm for 10 min and washed twice with phosphate buffered saline (PBS, pH 7). Stock solutions of isolates were prepared by resuspending the cell pellets in

50 mL of phosphate buffer saline. The initial populations of isolates ranged approximately from 10^5-10^6 CFU/mL, which were obtained by diluting the stock solution. Suspensions were refrigerated until needed, but not more than 6 h. Eighteen isolates were grown in Mueller-Hinton broth (Difco, Detroit, MI) with increasing amounts (4-64 ppm) of 10-different biocides according to the broth micro dilution method in 96-well plates. Serial twofold dilutions of biocides were prepared in distilled water[10]. Positive growth (diameter of precipitation ≥ 1 mm) was checked after incubation for 24 h at 30 ºC.

Reduction of the numbers of viable microorganisms count in the sample taking from cooling solution: To determine bactericidal activity of biocides in cooling solution, a cooling water sample was taken from a cooling tower of a petrochemical industry (Izmir, Turkey) before any antimicrobial agent was added. The initial populations of cooling water sample was counted using the plate count method and was found to be 3.9×10^4 cells/mL. To determine bactericidal activity against planktonic bacteria in cooling water of biocides, cooling water were transferred into tubes containing Mueller-Hinton broth with different biocide levels between 4 and 64 ppm. Contact time was 60 min. Bacterial cell enumeration was determined using the standard plating method for viable counts. Colony forming units (CFU) were counted after 48 h of incubation at 30 ºC.

Biocides tested: Star chem. Biocide Type 911; a liquid mixture based on quarternary ammonium salts and Star chem. Biocide Type 902; a liquid mixture based on quarternary ammonium salts (Jalan SS26/8, Taman Mayang Jaya, 47301 Petailing Jaya Selangor Darul Ehsan).

Henkel P3 Ferrocid® 8583; a liquid mixture based on organic N-heterocyclic compounds, Henkel P3 Ferrocid® 8585; a liquid mixture based on quarternary ammonium salts, Henkel P3 Ferrocid® 8581; a liquid mixture based on bromine and Henkel P3 Ferrocid® 8593; a liquid mixture based on quarternary ammonium salts (Henkel KGaA D-40191, Düsseldorf-Germany).

Concorde chimie Hydrobio 601; a liquid mixture based on polyaldehyde, Concorde chimie Hydrobio 602; a liquid mixture based on THPS, Concorde chimie Hydrobio 600; a liquid mixture based on quarter nary ammonium salts and Concorde chimie Hydrobio 620; a liquid mixture based on isothiazolones (43, Route de Ruaudin 72230 Arnage-France).

RESULTS AND DISCUSSION

Identification of the most exopolysaccharide producing bacteria isolated from cooling water: Based on light-microscopy observation, isolates were 8 gram-negative and 10 gram-positive. The API 20E, 20NE and API 50 CH Kits was used for physiological characterization of isolated strains. On the basis of these results 3 gram negative and 3 gram-positive bacteria were identified. The other bacteria could not be identified. Identified bacteria were *Pseudomonas fluorescens*, *Alcaligenes* spp., *Enterobacter agglomerans*, *Burkholderia cepacia*, *Aeromonas caviae*, *Pasteruella* spp. and 2 isolates of *Pseudomonas* spp. as gram negative and *Bacillus brevis*, *B. sphaericus*, *B. megaterium*, 3 unidentified spore forming *Bacilli* and 4 unidentified non spore forming *Bacilli* as gram-positive.

Effectiveness of various biocides on the most exopolysaccharide producing bacteria: Table-1 shows that 10 biocides were tried against exocellular polysaccharide producing 18 bacteria, which had been isolated from cooling water.

TABLE-1
MINIMAL INHIBITION CONCENTRATION OF VARIOUS BIOCIDES ON 18 EXOCELLULAR POLYSACCHARIDE PRODUCING BACTERIA ISOLATED FROM COOLING WATER OF A PETROCHEMICAL INDUSTRY

Biocide	Concentration of biocides					
	64 ppm	32 ppm	16 ppm	8 ppm	4 ppm	No effect
Star chem Biocide Type 911	9	–	–	–	–	9
Star chem Biocide Type 902	14	10	6	5	2	4
Henkel P3 Ferrocid 8583	16	11	5	1	1	2
Henkel P3 Ferrocid 8585	14	12	7	4	3	4
Henkel P3 Ferrocid 8581	–	–	–	–	–	18
Henkel P3 Ferrocid 8593	11	2	–	–	–	7
Concorde chem Hydrobio 601	2	–	–	–	–	16
Concorde chem Hydrobio 602	3	–	–	–	–	15
Concorde chem Hydrobio 600	15	8	4	1	–	3
Concorde chem Hydrobio 620	17	15	7	3	2	1

In present study, it was shown that Henkel P3 Ferrocid 8583, Henkel P3 Ferrocid 8585, Concorde chem Hydrobio 620 and Star chem Biocide Type 902 were more effective against isolated bacteria. While biocide concorde shows effectiveness against all isolates Henkel P3 Ferrocid 8581, Concorde chem Hydrobio 601 showed no or little effectiveness against isolated bacteria.

Henkel P3 Ferrocid 8581 is recommended for use to be mixed in water with an oxidant, such as hypochlorite or chlorine gas, to form the corresponding biocidally active hypobromous acid. Since we did not use it in such a way, any activity of it could not been determined.

The studies that were made with Henkel 8585 showed that the MIC value of 3 isolates is 4 ppm. However, such an effectiveness could not been shown in this concentration for other biocides.

In the second part of present study, 4 most effective biocides at different concentrations against biofilm bacteria the values of the 4 most effective biocides, which had been selected, determined at the reduction of the numbers of viable microorganisms count in the sample taking from cooling solution of a petrochemical industry. Although biocides were tried on high concentrations in present work, the less effectiveness of biocides against biofilm bacteria is consistent with results found in the literature[5,11].

Fig. 1 shows percentage reduction values in microorganisms' count of the 4 biocides in the sample taken from cooling water before any antimicrobial agent was added. It was demonstrated that the most percentage reduction of 4 ppm concentrations was in Henkel P3 Ferrocid 8585 and the least percentage reduction value was in Concorde chem Hydrobio 620.

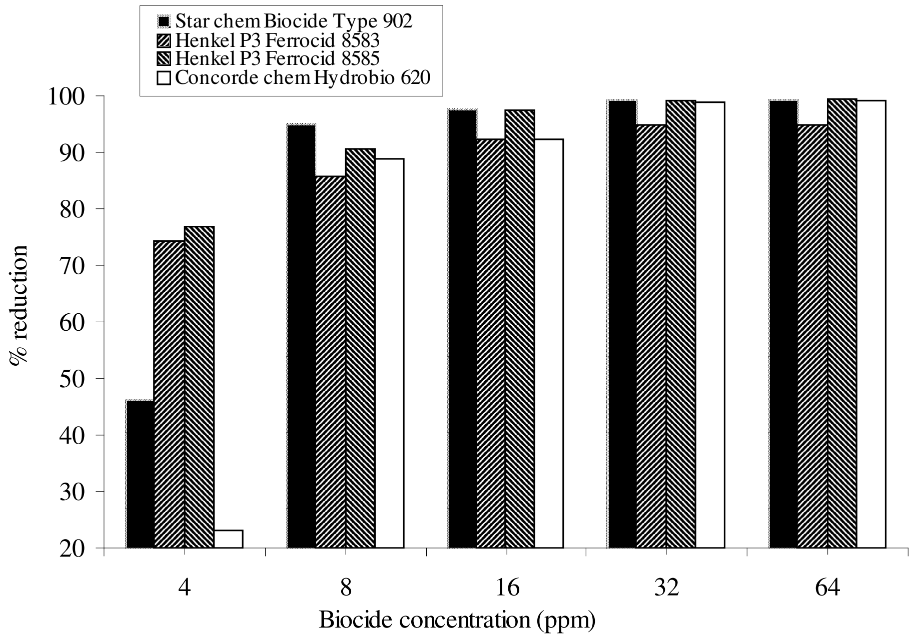

Fig. 1. Percentage reduction of the counts of viable microorganisms by selected biocides in the sample taking from cooling solution

The number of colony forming units was reduced by 99 % (4.59-log inactivation of the heterogeneous plate count bacteria) after 30 min in 64 ppm for 3 disinfectants except for Henkel P3 Ferrocid 8583, which was reduced by 94.87 % (4.57-log inactivation of the heterogeneous plate count bacteria).

If compared, the effectiveness of biocide ferrocid 8585 on pure culture studies is higher than the studies of biocide ferrocid 8585 and cooling solution. However, in cooling water studies, there is not a big difference among the selected four biocides with the effectiveness levels. Interestingly, the results of this study clearly demonstrate that the most effectiveness biocide against isolated bacteria did not have the same effectiveness when it was tried with cooling solution. Especially, due to isolated bacteria are the bacteria producing exocellular polysaccharide, it is thought that they are different from planktonic bacteria of cooling water. Because of these exocellular polysaccharide producing bacteria have been of a higher importance in the production of a biofilm, it is even more important that biocide is more effective against exocellular polysaccharide producing bacteria.

Conclusion

In cooling water systems, the vast majority of bacteria are found in sessile, biofilm communities, rather than as planktonic organisms in the liquid phase. Biofilm organisms especially produce exocellular polysaccharide. Disinfectants are the lesser effectiveness against biofilm bacteria than planktonic bacteria because of composition of the outer layers of the biofilm matrix, the blockage of inner channels of the biofilm structure and the existence of high density microbial aggregates in the inner region of biofilms which are more resistant to biocidal action. Generally, biofouling monitoring of the cooling water is done by counting the planktonic cells in suspension. It is well known that it is possible to kill all planktonic cells in cooling water and still have an active biofilm on an associated surface. The location of the biofilm on a surface and its structure, resist the action of many biocides so that any monitoring system must assess biofilm accumulation *in situ* on a surface.

The trade biocides, which have similar active matter, used in present work showed different activity against biofilm microorganisms of cooling water of a petrochemical industry.

The use of these biocides and other chemicals for the control of biofilms in cooling water systems tended to be indiscriminate and these chemicals were environmentally detrimental. Recently, with greater public concern for the environment, there has been a move towards the use of minimal quantities of additives by effective dosing programmes and more reliable monitoring. From this point of view, a biocide, which will be used for cooling water, must be well selected.

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