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ORIGINAL ARTICLE

The antimicrobial mechanism of electrochemically activated water against *Pseudomonas aeruginosa* and *Escherichia coli* as determined by SDS-PAGE analysis

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anolyte, electrochemically activated water, *Escherichia coli, Pseudomonas aeruginosa,* SDS-PAGE.

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Abstract

Aims: To expose bacteria to anolyte and subsequently investigate the effect of anolyte on the protein profiles of treated bacteria.
Methods and Results: Proteins were extracted from bacteria treated with different concentrations of anolyte and analysed using SDS-PAGE. Fewer and more faint protein bands were observed for concentrated halide anolyte treated bacteria when compared to untreated bacteria while extra protein bands were observed for bacteria exposed to dilute concentrations.
Conclusions: The undiluted and the 10^{-1} dilution of halide derived anolyte was effective in killing the test bacteria. Anolyte caused bacterial death by complete destruction of proteins or by causing oxidative stress which resulted in protein fragmentation.
Significance and Impact of the Study: The results of this study provide information on the antimicrobial mechanism of anolyte on other bacteria for which the information is currently unavailable.

Introduction

Resistance of bacteria to most of the commonly used biocides has serious economic and environmental implications in various applications. These include cooling water, papermaking, medical implants, drinking water distribution and food processing (Cloete 2003). This necessitated the search for novel effective and environmentally friendly alternatives. Electrochemically activated (ECA) water is one of the developed alternatives.

The detailed description of the ECA water production process has been outlined by various researchers (Leonov 1997; Marais and Brözel 1999; Rogers *et al.* 2006). Two types of solutions are produced by electrochemical activation. Catholyte, has a negative oxidation reduction potential (ORP) and anolyte has a positive ORP (Marais and Brözel 1999; Al-Haq *et al.* 2002). The anolyte has antimicrobial effects and has been used in studies for the control of microbial growth in various fields (Marais and Brözel 1999; Selkon *et al.* 1999; Middleton *et al.* 2000; Marais and Williams 2001; Rogers *et al.* 2006; El karim *et al.* 2007). Thantsha and Cloete (2006) indicated effective removal and control of biofilms related to a water distribution system and industrial cooling water using ECA water. Yang *et al.* (1999) and Russell (2003) investigated the ability of ECA water to reduce microbial contaminants on eggs and poultry carcasses. Results from the above mentioned studies confirm the ability of ECA water to kill micro-organisms.

The ORP values of anolyte ranged between +700 and +1000 mV (Marais and Brözel 1999; Al-Haq *et al.* 2002; Deza *et al.* 2003). ORP is a measure of a solution's oxidizing and reducing activity (Banhidi 1999). There was a direct correlation between the oxidant residual, 'oxidizing potential' of the water and the ORP mV reading (Kelly 2004). The higher positive ORP values indicate a strong oxidizing power (Park *et al.* 2004). Exposure of bacteria to oxidative compounds from either endogenous or exogenous sources induces oxidative stress (Rowbury and Goodson 1999; Shackelford *et al.* 2000). Research indicated that an ORP value of +650 mV to 700 mV will kill bacteria within a few seconds (Suslow 2000; Zinkevich *et al.* 2000). It was therefore, hypothesized that the bactericidal activity of ECA water was attributed to ORP values

exceeding 750 mV (Kim *et al.* 2000; Park *et al.* 2004) and the concomitant destruction of proteins.

Although the ECA solutions have antimicrobial properties, the exact mechanism by which bacteria are killed is unknown (Al-Haq *et al.* 2002). Biocides kill micro-organisms through the targeting of several structures or macromolecules or biochemical pathways (Denyer and Stewart 1998; McDonnell and Russell 1999; Russell 1999). The high oxidation potential of ECA solutions may inhibit microbial growth through oxidation of sulfhydryl compounds on cell surfaces and other key metabolites (Park *et al.* 2004). ECA solutions have the ability to eliminate or destroy bacterial endospores (Rogers *et al.* 2006). Since they are able to reduce endospores, they may have the ability to affect the spore coat for it to inactivate the spore. The structure of the endospore, especially the spore coat, is made up of proteins (Rogers *et al.* 2006). Thus if ECA water affects the spore proteins, then possibly, death of vegetative cells could be due to contact between the ECA solutions and cellular proteins. Zinkevich *et al.* (2000) investigated the mechanism of action of ECA water against *E. coli*. They indicated total destruction of nucleic acids and proteins within 5 min of exposure.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is a powerful tool to dissociate proteins into individual chains and separate them according to their molecular weight (Jason and Rydén 1998; Walker 2002). SDS-PAGE is therefore an ideal technique to use for demonstrating antimicrobial effectivity and has previously been used to study resistance mechanisms in bacteria (Brözel and Cloete 1993). The main objective of this study was to determine the effect of anolyte on the bacterial cell proteins, using SDS-PAGE in order to elucidate the mechanism of antimicrobial action.

Materials and methods

Bacterial cultures

Pseudomonas aeruginosa, *Staphylococcus aureus*, *Bacillus subtilis* (vegetative) and *Escherichia coli* used in this study were obtained from the Department of Microbiology and Plant Pathology Culture collection, University of Pretoria. Cultures were maintained on Nutrient Agar plates and subcultured every 2 weeks. Gram staining was used to check purity. Anolytes were supplied by Radical Waters Ltd, Johannesburg, South Africa. These solutions were stored at 4°C and used within 24 h.

Production of anolyte

Sodium chloride stock solution (2·5 g l^{-1}) was constituted in softened water with alkalinity of <20 mg l^{-1}. This stock solution was fed into the two reactor device at a rate of 700 ml min^{-1}. The reactor system was preset to produce anolyte with near neutral pH (pH 6·8–7), and the power supply was preset at 12 V and 5 A per electrode reactor. The physicochemical properties of the anolyte were measured (Table 1).

Table 1 Properties of the diluent and solutions used for treatment of bacteria

	Solutions				
		Halide (NaCl) anolyte			
Parameter	Ringers's solution	Undiluted	10^{-1}	10^{-2}	10^{-3}
OR* (mV)	234	902	700	593	365
pH	6·9	6·8	6·4	6·4	6·6
EC (mS)	5·38	3·04	4·58	4·44	2·35
Temperature (°C)	25·4	24·0	24·4	24·5	25·1

*ORP, oxidation reduction potential; EC, electrical conductivity.

Properties of anolyte solutions

The oxidation reduction potential (ORP), electrical conductivity (EC), temperature and pH were measured before the solutions were used. A waterproof ORPScan (double junction) with replaceable double junction silver-chloride (AgCl) electrode and 1 mV resolution was used to measure ORP of the anolyte solutions. Electrical conductivity and temperature were measured using Waterproof ECScan with replaceable electrode, temperature display and 0·01 mS resolution. The pH was measured using a waterproof pHScan 2 tester. All the probes were from Thermo Fisher Scientific, Inc., Eutech Instruments Pte Ltd., Singapore. Levels of oxidizing agents present in anolyte were determined using the respective Spectroquant test kits [Merck Chemicals (Pty) Ltd, Modderfontein, South Africa] according to the manufacturer's instructions.

Determination of minimum inhibitory concentration

Cultures were grown on nutrient agar (NA) plates and incubated at 37°C for 24 h. An overnight culture on NA plate was suspended with freshly prepared 0·25 strength Ringer's solution [Merck Chemicals (Pty) Ltd]. 1 ml of each bacterial suspension was added to four different tubes, each containing a different concentration of anolyte [undiluted, 10^{-1}, 10^{-2} and 10^{-3} (anolyte: Ringer's solution)]. The control tube contained 1 ml of the bacterial suspension and 9 ml of 0·25 strength Ringer's solution. The tubes were vortexed after addition of the culture and then 100 μl was taken from each tube as 0 h sample. All the test tubes were incubated at room temperature for

6 h and then another sample was taken for bacterial counts. No neutralizer was added due to dilution of the sample which would have eliminated the antimicrobial effect in solution (Middleton *et al.* 2000; Cloete 2003). The 100 μl aliquots were serially diluted (from 10^{-1} to 10^{-6}) in 0·25 strength Ringer's solution and spread-plated on NA plates in duplicates. The plates were incubated at 37°C for 24 h. The lowest concentration of anolyte that resulted in total elimination of bacteria (no growth on plates) was taken to be the minimal inhibitory concentration (MIC).

Determination of minimum exposure time

A 24 h culture on NA plate was suspended in freshly prepared 0·25 strength Ringer's solution. 1 ml of the bacterial suspension was added to 9 ml of the MIC of the anolyte determined above. For the control, 1 ml of the bacterial suspension was added to 9 ml of 0·25 strength Ringer's solution. Tubes were incubated at room temperature for 6 h. 100 μl samples were taken from each tube immediately after mixing (0 min) and after 5, 10, 15, 20, 25, 30 and 60 min. Serial dilutions of samples were prepared and then 100 μl of appropriate dilutions were spread out on NA plates in duplicate. The plates were incubated at 37°C for 24 h. The shortest exposure time that was bactericidal was taken as the minimum exposure time.

Sample preparation for SDS-PAGE

P. aeruginosa and *E. coli* cells were grown on NA plates for 24 h at 37°C and suspended in 20 ml of 0·25 strength Ringer's solution. The suspensions were transferred into BD Falcon™ 50 ml tubes (BD Biosciences, New Jersey, USA) and centrifuged for 10 min at 10·5 ***g*** using Eppendorf Centrifuge 5804R (Merck Chemicals (Pty) Ltd). The supernatant was discarded, and the pellet was washed four times with 20 ml phosphate buffer of pH 6·8 (112·5 ml of 0·2 mol l^{-1} $NaH_2PO_4{\cdot}2H_2O$ + 137·5 ml of 0·2 mol l^{-1} $Na_2HPO_4{\cdot}12H_2O$). The pellet was resuspended in 1 ml of phosphate buffer and then transferred to a preweighed Eppendorf tube. Cells were centrifuged for 11 min at 10·5 ***g*** and the supernatants were discarded. The mass of the pellet was determined by weighing the tubes. 100 μl of 20% SDS were mixed with 900 μl of STB (sample treatment buffer) for use with proteins. 100 μl of SDS-STB solution was added to the pellet and heated for 3 min at 96°C. Cells were lysed by sonication for about 5 s using an ultrasonic homogenizer 4710 (Cole-Palmer, Chicago) with an output (40 W) using 15 pulses. Another 100 μl of SDS-STB mixture was added and cells were centrifuged for 11 min at 10·5 ***g***. The supernatant was transferred to the sterile Eppendorf tube and stored according to Ehlers (1997).

SDS-PAGE protein analysis

SDS-PAGE was performed by the method described by Hames (1990), modified according to Jason and Rydén (1998). Proteins were separated on gels (1·5 mm thick and 125 mm long), which were run in a Hoefer SE 600 dual cooled vertical slab unit (Hoefer Scientific Instruments, San Francisco, CA, USA). The separation gel (12%, 1·5 mol l^{-1} Tris-HCl pH 8·66 with conductivity of 17·5 mS) and the stacking gel (5% 0·5 mol l^{-1} Tris-HCl pH 6·68 with conductivity 28–33·5 mS) were prepared from monomer solution containing 29·2% (m/v) acrylamide (BDH Electran, Lutterworth, UK) and 0·8% (m/v) N^1-N^1-bismethyleneacrylamide (BDH Electran). After pouring the separation gel, it was overlayed with butan-1-ol. The separation gel was allowed to polymerize for 30 min. After polymerization, butan-1-ol was removed, and the gel was washed three times with distilled water before addition of stacking gel. The gel was then covered with plastic and the stacking gel was allowed to polymerize overnight. Samples were boiled for 10 min at 100°C and then 2 μl of each sample was loaded on the gel. Electrophoresis was performed at a constant current of 22 mA through the stacking gel (for 1 h 45 min), and at 32 mA through the separation gel (for 3 h 15 min) at 20°C.

Staining of proteins

Gels were stained in a solution containing Coomassie Blue stock solution, methanol and acetic acid in the ratio 1 : 4 : 2 for 1 h at room temperature. Then the gels were destained overnight in a solution containing methanol, acetic acid and double distilled water in the ratio 5 : 2 : 20 at room temperature. Then the gel was scanned using an Amersham Pharmacia Biotech ImageScanner.

Results

Properties of anolyte solutions

Table 1 shows the properties of the solutions used. Anolyte had a positive ORP value of 902 mV, attributed to presence of oxidizing agents in solution (Table 2). A

Table 2 Oxidants present in anolyte and their concentrations

Oxidizing agent	Concentration (mg l^{-1})
Free chlorine	180
Total chlorine	180
Ozone	120
ClO_2	5
H_2O_2	Undetectable

Table 3 Counts of bacteria after exposure to different concentrations of NaCl anolyte

	Bacterial species							
	B. subtilis		*S. aureus*		*P. aeruginosa*		*E. coli*	
	Time of exposure (h)							
	0	6	0	6	0	6	0	6
Biocide	Bacterial counts (CFU ml^{-1})							
Control	$1{\cdot}67 \times 10^{7}$	$2{\cdot}14 \times 10^{7}$	$>3{\cdot}00 \times 10^{8}$	$>3{\cdot}00 \times 10^{8}$	$>3{\cdot}00 \times 10^{8}$	$1{\cdot}68 \times 10^{8}$	$>3{\cdot}00 \times 10^{8}$	$>3{\cdot}00 \times 10^{8}$
Undiluted	0	0	0	0	0	0	0	0
10^{-1}	$1{\cdot}42 \times 10^{5}$	0	0	0	0	0	0	0
10^{-2}	$2{\cdot}40 \times 10^{6}$	$1{\cdot}88 \times 10^{4}$	$6{\cdot}40 \times 10^{6}$	$2{\cdot}14 \times 10^{8}$	$1{\cdot}85 \times 10^{8}$	$8{\cdot}40 \times 10^{8}$	$2{\cdot}00 \times 0^{8}$	$1{\cdot}80 \times 10^{8}$
10^{-3}	$2{\cdot}50 \times 10^{6}$	$>3{\cdot}00 \times 10^{8}$	$6{\cdot}40 \times 10^{8}$	$6{\cdot}60 \times 10^{8}$	$8{\cdot}40 \times 10^{8}$	$9{\cdot}51 \times 10^{8}$	$1{\cdot}80 \times 10^{8}$	$>3{\cdot}00 \times 10^{8}$

CFU, colony forming units.

reduction in the original ORP value of undiluted anolyte solutions was observed upon dilution. This decrease in the ORP reflected the decrease in the biocidal effect of the anolyte (Tables 2 and 3). The pH of the solutions did not vary much upon dilution of the solutions. Electrical conductivity of the anolyte fluctuated upon dilution (Table 1).

Determination of minimum inhibitory concentration of halide anolyte

The undiluted anolyte eliminated all test organisms immediately upon exposure (Table 3). The 10^{-1} anolyte dilution also completely eliminated all the test organisms immediately upon exposure, except for *B. subtilis* whose numbers were reduced from $1{\cdot}67 \times 10^{7}$ to $1{\cdot}42 \times 10^{5}$ CFU g^{-1} immediately after exposure (Table 3). However, all *B. subtilis* cells were killed by this concentration of anolyte after an exposure period of 6 h. The 10^{-2} anolyte dilution reduced the viable numbers of all the test organisms while no biocidal effect was observed for 10^{-3} anolyte dilution on any of the test organisms (Table 3). It was, therefore, concluded that the MIC for the halide anolyte was a 10^{-1} dilution.

Table 4 Bacterial counts after exposure to 10^{-1} dilution (MIC) of NaCl anolyte (ORP 700 mV) for varying times

	Bacterial species			
	Bacterial counts (CFU ml^{-1})			
Exposure time (min)	*B. subtilis*	*S. aureus*	*P. aeruginosa*	*E. coli*
0	$>3{\cdot}00 \times 10^{8}$	$>3{\cdot}00 \times 10^{8}$	$>3{\cdot}00 \times 10^{8}$	$>3{\cdot}00 \times 10^{8}$
5	$>3{\cdot}00 \times 10^{6}$	0	0	0
10	$3{\cdot}20 \times 10^{5}$	0	0	0
15	$1{\cdot}90 \times 10^{5}$	0	0	0
20	$2{\cdot}25 \times 10^{4}$	0	0	0
25	$2{\cdot}75 \times 10^{3}$	0	0	0
30	$6{\cdot}00 \times 10^{2}$	0	0	0
60	0	0	0	0

CFU, colony forming units.

Determination of minimum exposure time

When the test organisms were exposed to the MIC of the halide anolyte, a 100% kill effect after only 5 min of

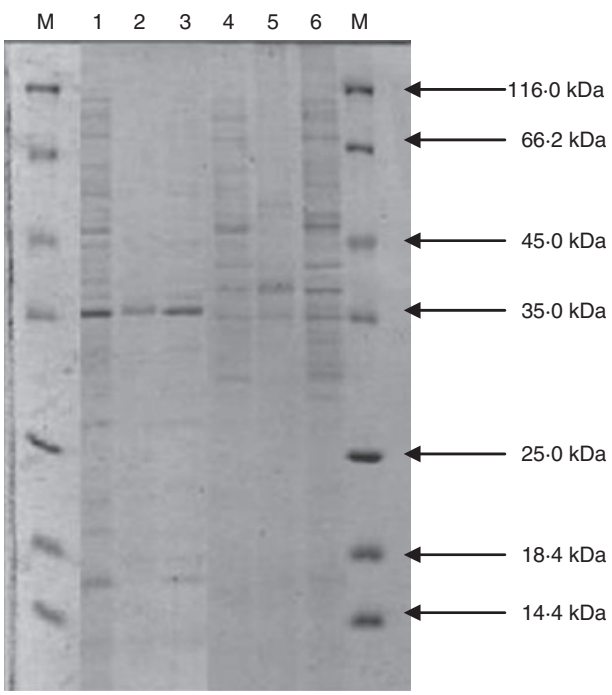

Figure 1 SDS-PAGE whole protein profiles from bacteria treated and untreated with halide anolyte. Lanes 1 and 4 are untreated cells of *P. aeruginosa and E. coli*, respectively. Lanes 2 and 5 are proteins from *P. aeruginosa and E. coli*, respectively, treated with 10^{-1} anolyte dilution; lanes 3 and 6 *P. aeruginosa and E. coli*, respectively, treated with 10^{-2} anolyte dilution. M lanes represent the molecular weight marker.

exposure was observed for all the test organisms except *B. subtilis*, which were reduced from $>3{\cdot}00 \times 10^8$ to $>3{\cdot}00 \times 10^6$ CFU ml^{-1} (Table 4). These numbers decreased further to $6{\cdot}00 \times 10^2$ CFU ml^{-1} after 30 min exposure period, and ultimately to 0 after 60 min (Table 4). This result suggests that *B. subtilis* was the most resistant to anolyte exposure. The minimum exposure period for efficient bactericidal effect of the MIC of halide anolyte was 1 h for *B. subtilis* and 5 min for all the other test organisms.

Protein analysis of bacterial cells after treatment with halide anolyte

Pseudomonas aeruginosa

The protein profiles of untreated bacteria differed from those of anolyte treated bacteria. The protein profiles of bacteria treated with different concentrations of anolyte were also different. Several protein bands were observed for untreated *P. aeruginosa* (Fig. 1, lane 1) while fewer bands were observed for the same cells treated with anolyte. There was a single band for 10^{-1} anolyte treated cells. There were three bands for bacteria treated with 10^{-2} dilution (Fig. 1, lanes 1, 2 and 3). The intensities of the protein bands for treated bacteria were lower than for untreated cells, and the intensities of the 10^{-1} anolyte treated cells was lower than for 10^{-2} anolyte treated cells (Fig. 1, lanes 2 and 3).

Escherichia coli

The results obtained for *E. coli* differed from those of *P. aeruginosa.* Similar to the observations made for *P. aeruginosa*, there were fewer protein bands from *E. coli* cells treated with 10^{-1} dilution than those of untreated cells (Fig. 1, lanes 4 and 5). However, the 10^{-2} dilution reduced the numbers of viable cells and also resulted in more protein bands (Fig. 1, lane 6).

Discussion

Anolyte decreased the numbers of viable bacteria and the biocidal effect decreased with progressive dilution of anolyte and a concomitant reduction in the ORP. There was a reduction in the number of protein bands after the bacterial strains were exposed to undiluted anolyte. This suggests that anolyte may have caused the death of the bacteria through the destruction of cellular proteins. These results are in agreement with the findings of Zinkevich *et al.* (2000) who also observed the disappearance of protein bands after exposing *E. coli* to an anolyte solution with an ORP of 1000 mV. Oxidizing compounds present in anolyte probably cause destruction of proteins by breaking down the covalent bonds in proteins (Zinkevich *et al.* 2000). The 10^{-2} anolyte dilution destroyed proteins of *P. aeruginosa* but in *E. coli* it resulted in appearance of even more protein bands when compared to the protein profile of the untreated bacteria. The extra protein bands were of both low and larger molecular weight than the native proteins present in untreated bacteria. Low molecular weight protein bands probably resulted from fragmentation the proteins into smaller peptides (Janig *et al.* 2005; Li *et al.* 2005). Presence of dilute anolyte caused an unfavourable environmental condition which induced a stress response in bacteria. The level of stress response varies with the type of organism and the type of environment (Kochhar and Kochhar 2005). During exposure to stressful conditions, bacteria synthesize and replace damaged proteins from mainly recycled amino acids, and this may give rise to the altered profiles from the native proteins (Davies 2001). Also, stress initiates the activation of a variety of defence genes which encode for the scavengers of reactive radical and other stress related proteins (Aertsen and Michiels 2004; Kochhar and Kochhar 2005). This condition may account for the presence of the additional, large molecular weight protein bands as evidenced.

Conclusions

The undiluted halide derived anolyte was effective in killing all the test bacteria immediately upon exposure. The same killing effect was observed for 10^{-1} dilution of anolyte on all the test bacteria except *B. subtilis*, which exhibited the most resistance. The exposure time for total elimination of *B. subtilis* by 10^{-1} anolyte dilution was 6 h. Anolyte killed *P. aeruginosa* and *E. coli* bacteria by interfering with their protein composition, either completely or partially degrading proteins due to oxidative stress.

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