Romanian Biotechnological Letters *Vol. 20, No. 1, 2015*

ORIGINAL PAPER

Extermination of bacteria in hide-brine curing liquors using combined alternating and direct electric current applications plus bronopol

Received for publication, July 06, 2014
Accepted, December 20, 2014

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Abstract

Microbial spoilage of hide usually results from the failure to inactivate destructive microorganisms during hide brine curing. To compensate for this problem, an effective inactivation method to be used in the leather industry was researched. Three different experiments were performed with the mixed culture of six hide bacteria to examine: ***1)*** *the effect of only bronopol on mixed culture;* ***2)*** *the effect of 188 μA/m^2 DC treatment applied together with 251 μA/m^2 AC treatment followed by 1 g/L of bronopol on mixed culture;* ***3)*** *the effect of 188 μA/m^2 DC treatment applied alternately with 251 μA/m^2 AC followed by a rest break of 3 h, and then renewed AC/DC application followed by addition of 1 g/L bronopol to mixed culture. The mixed cultures were completely killed with bronopol in 10 h in experiment 1. The mixed culture damaged by the electric treatments was killed by bronopol in 8 h in experiment 2. The mixed culture damaged by two cycles of the electric treatments was killed by bronopol in 3 h in experiment 3. In conclusion, combinatory usage of both two cycles of electric treatment and bronopol may be applied in hide-brine curing process to inactivate proteolytic and lipolytic bacteria.*

Keywords: Electrochemical disinfection, electric current, bronopol, leather, brine curing process, bacteria

1. Introduction

Hides, which have high economic value, are a by-product of the meat industry. In the leather industry, the most important problem encountered is the inadequate preservation of raw hide after the animal is slaughtered. The animals may be contaminated by the bacteria found in the air, faeces, animal feeds, dust and on animal's skin (S. DAHL [1], M. BIRBIR & al. [2]). If hide is not preserved well after slaughtering process, it may be damaged by the hydrolytic enzymes of microorganisms during storage. Therefore, adequate preservation of hides is crucial in slaughterhouses and tanneries. Hides are usually preserved with salt, boric acid and antimicrobial agents. If the bacterial populations on the salted hides cannot be exterminated using effective preservation methods, destructive activities of bacteria will also continue in the soaking process (R. RANGARAJAN & al. [3], D. BERBER & al. [4], D. BERBER & al. [5]).

In our previous study, despite the salt curing process of hides, fairly high numbers of bacteria (10^4-10^8 CFU/g) were recovered from the 36 salted hides imported mostly (83%)

from Greece, England, United States, Serbia, Bulgaria, Russia, Africa and Australia and collected from Turkey (17%). Proteolytic and lipolytic bacteria were isolated from 97% of the hide samples in high numbers (D. BERBER & al. [5]).

In another investigation, 47 different Gram-positive bacterial species belonging to 12 different genera (*Aerococcus, Aneurinibacillus, Bacillus, Brevibacillus, Enterococcus, Geobacillus, Kocuira, Lactococcus, Paenibacillus, Streptococcus, Staphylococcus* and *Virgibacillus*) and 46 different Gram-negative bacterial species belonging to 21 different genera (*Acinetobacter, Aeromonas, Alcaligenes, Burkholderia, Citrobacter, Comamonas, Edwardsiella, Enterobacter, Escherichia, Hafnia, Klebsiella, Mannheimia, Pasteurella, Proteus, Pseudomonas, Salmonella, Serratia, Sphingomonas, Stenotrophomonas, Vibrio* and *Yersinia)* were isolated and identified from 10 salted hides preserved in different countries. More than half of these isolates showed proteolytic and lipolytic activities which might reduce leather quality. These results showed that the presence of bacteria in high numbers on salted hides was a common problem worldwide. Furthermore, traditional preservation technique was ineffective for removing these bacteria from the salted hides (E. ASLAN & al. [6], E. ASLAN & al. [7]).

Microorganisms grown on the salted hides may cause hair slip, discoloration, serious grain peeling, fiber destruction, odor, looseness, weakness and holes in leather (S. DAHL [1], M. BIRBIR & al. [2], H. ANDERSON [8], B.M. HAINES [9], J.J. TANCOUS [10]). It has been known that bacterial cells have a wide variety of abilities to survive and grow in the presence of antibacterial agents (A. BALOWS [11], S.S. BLOCK [12]). Therefore, it is not easy to kill harmful bacteria on the hides with commonly used preservation methods in the leather industry. Because of successful defense mechanisms of Gram-positive and Gram-negative bacteria, researchers have been obliged to develop different inactivation methods to kill bacteria.

Electric current has been proven to be an effective weapon against several resistance capacities and virulence factors of microorganisms. Electric current was used for disinfection of titanium implants covered by biofilms formed by Gram-negative *Escherichia coli* (7.0-7.5 log CFU/ml). The colonized implants were treated with 2 mA, 5 mA, 7.5 mA or 10 mA of direct electric current for 15 min. Direct electric current (DC) of 7.5 mA eliminated the *Escherichia coli* biofilm from the dental implants.

In addition, electric current was utilized in transdermal transport of chemicals. Transdermal iontophoresis has been intensively used in delivery of anti-inflammatory drugs and other agents (C.T. COSTELLO & al. [13]). It was demonstrated that electric current can improve the absorption capacity of the skin through numerous feasible methods, including an electrophoretic driving force, an electro-osmotic driving force, and transiently increased skin permeability (M.J. PIKAL [14], M.R. PRAUSNITZ & al. [15]).

Previous studies indicated that antimicrobial agents and electric current acted synergistically to kill microorganisms (J.W. COSTERTON & al. [16], A.E. KHOURY & al. [17], S.A. BLENKINSOPP & al. [18], J. JASS & al. [19]). Researchers explained that the efficacy of antibiotics was enhanced through the application of electric currents (J.W. COSTERTON & al. [16], S.A. BLENKINSOPP & al. [18], J. JASS & al. [19]). Kathon (isothiazalone, 1.5%), glutaraldehyde (glutaraldehyde, 25%), and quaternary ammonium compound (dimethyl ammonium chloride, 50%) exhibited enhanced action when applied against *Pseudomonas aeruginosa* biofilm within a low-strength electric field (12 V/cm) with a low current density (2.1 mA/cm^2). Researchers furthermore mentioned that these antimicrobial agents were bactericidal within 24 h when utilized together with the electric treatment.

Notwithstanding reports that electrochemical inactivation method can eradicate different species of microorganisms in diverse industries (J.W. COSTERTON & al. [16], A.E. KHOURY & al. [17], S.A. BLENKINSOPP & al. [18], J. JASS & al. [19], M. BIRBIR & al. [20], Y. BIRBIR & al. [21]), the combinatory application of DC electricity followed by AC and bronopol on the mixed culture of Gram-positive (*Staphylococcus cohnii* and *Enterococcus faecium*), Gram-positive endospore-forming bacteria (*Bacillus pumilus*) and Gram-negative bacteria (*Enterobacter cloacae, Vibrio fluvialis* and *Pseudomonas luteola*) in the hide-brine curing liquor consisting of organic matter and 25% NaCl is yet to be revealed. The aim of this investigation was to determine the effect of DC treatment applied together with AC in a sequential manner and bronopol. Therefore, different three experiments were performed with the mixed culture of hide bacteria to examine: **1)** the effect of 1 g/L bronopol on the mixed culture during 10 h storage; **2)** the effect of 188 $\mu A/m^2$ DC treatment applied together with 251 $\mu A/m^2$ AC treatment followed by 1 g/L of bronopol on the mixed culture, then followed by 10 h storage of the mixed culture; **3)** the effect of 188 $\mu A/m^2$ DC treatment applied alternately with 251 $\mu A/m^2$ AC for 20 min followed by a rest break of 3 h, and then renewed AC/DC application for 12 min followed by addition of 1 g/L bronopol to the mixed culture before allowing 7 h rest.

3. Materials and Methods

Test microorganisms and antibacterial agent

Experiments were carried out with mixed culture of six hide microorganisms, i.e. Gram-negative (*Enterobacter cloacae, Vibrio fluvialis* and *Pseudomonas luteola*), Gram-positive (*Staphylococcus cohnii* and *Enterococcus faecium*) and Gram-positive endospore-forming bacteria (*Bacillus pumilus*), isolated and identified by API test kits (BioMérieux, Inc, France) in the previous studies (E. ASLAN & al. [6], E. ASLAN & al. [7]). These isolates were used as the test microorganisms due to their occurence in high frequency on the salted hides. The *Pseudomonas luteola, Enterococcus faecium, Staphylococcus cohnii* and *Bacillus pumilus* strains were both protease and lipase positive, while *Enterobacter cloacae* and *Vibrio fluvialis* were protease positive but lipase negative (Y. BIRBIR & al. [22]). The agent containing 2-bromo-2-nitropropane-1,3-diol was used as a test bactericidal agent. Bronopol was provided by Buckman International, Memphis, TN.

Inactivation of the test microorganisms and mixed culture

Test microorganisms were separately grown in Nutrient Broth (NB) (Merck, Darmstadt, Germany) at 37°C for 24 h. Then, each of these bacterial cultures was separately suspended in sterile 0.9% NaCl to a final density of 10^8 CFU/ml. Then, the mixed culture of the test isolates was prepared from these physiological saline solutions.

In determining the inactivation effect of different treatments against the mixed culture of hide microorganisms, we took account of different exposure times and different applications. Hence, three different experiments were conducted in the present research. For each experiment, 20 ml of the bacterial suspension was poured into the treatment system containing 180 ml of NB and 25% NaCl. Prior to each experiment, a 100 ml quantity of the test medium was extracted from the system and diluted to 10^{-4} with sterile 0.9% NaCl. Then, the diluted suspension was spread over Nutrient Agar (NA) (Merck, Darmstadt, Germany) and incubated at 37°C for 24 h. The colonies developed on NA medium were counted. Each

test medium's temperature and pH was adjusted to 20°C and respectively 7 before initiating the experimental procedure.

Experiment 1 **–** The test medium containing the mixed culture and 1g/L of bronopol was utilized in experiment 1. The bacteria in the test medium were exposed to the test agent for 10 h at room temperature. Then, 100 µl of the bacterial suspension were taken from the test medium at intervals ranging from 1 h to 10 h during the experiment, and diluted to 10^{-2} and 10^{-4} in sterile physiological saline solution. Afterwards the direct and diluted solutions were spread over NA and incubated at 37°C for 24 h. Finally, the colonies on the agar surface were counted.

Experiment 2 **–** The test medium containing the mixed culture was utilized in experiment 2. The electrochemical disinfection system was used in experiments 2 and 3. This system was constructed of a glass beaker containing two internally attached platinum wire electrodes submerged in the NB medium composed of 25% NaCl and organic matters. Each electrode was 1 mm in diameter and 80 mm in length, at a separation of 40 mm. The electrodes were connected to a variable alternating current source (VARIAC), (Input = 220 V, f = 50 Hz, VA = 2250 VA), with AC variable output voltage range of 0-220 V and alternating power output electric current level 0-1257 µA/m^2. The apparatus featured an AC/DC main switch for AC and DC output power supply selection, DC power output level with variable output voltage range of 0-200 V and current range of 0-1257 µA/m^2 (J.C. PARK & al. [23], Y. BIRBIR & al. [24]). Firstly, 188 µA/m^2 DC was applied for 2 min, and then 251 µA/m^2 AC treatment for an additional 2 min. The test medium was kept for 4 min at room temperature following application of sequentially paired DC and AC treatments. This method was implemented five times (totally 20 min of DC and AC treatments and 16 min without any treatment) until the temperature of the medium rose to 30°C. Afterwards 1 g/L of the test agent was put into the medium, which was left for 10 h at room temperature. Aliquots of 100 µl were taken from the test medium at intervals of 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 min during DC and AC treatments, and at intervals ranging from 1 h to 10 h after the test agent was put into the test medium and diluted to 10^{-2} and 10^{-4} in sterile physiological saline solution. Then, the direct and diluted solutions were spread over NA and incubated at 37°C for 24 h. Later, the colonies on the agar surface were counted.

Experiment 3 **–** The test medium containing the mixed culture was used in this experiment. Firstly, 188 µA/m^2 DC was applied for 2 min, and then 251 µA/m^2 AC treatment for an additional 2 min. The test medium was kept for 4 min at room temperature following application of sequentially paired DC and AC treatments. This method was implemented five times (totally 20 min of DC and AC treatments and 16 min without any treatment) until the temperature of the medium rose to 30°C. Afterwards, the medium was left for 3 h without any treatment. Then, 188 µA/m^2 DC was applied for two min and continued with 251 µA/m^2 AC treatment for two more min. Again, the test medium was left for four min without any electric current treatments after sequential application of DC and AC treatments. Then, this electric treatment was repeated three times (totally 12 min DC/AC treatments and eight min without any treatments) until the temperature of the medium rose to 35°C. One g/L of bronopol was added into the medium and this medium was left for 7 h at room temperature. Aliquots of 100 µl were taken from this medium at two-minute intervals up to 20 minutes during the first DC/AC treatments, at intervals of 1 h, 2 h, 3 h during the first rest period at room temperature, intervals of 2, 4, 6, 8, 10 and 12 min during the second DC/AC treatments, at intervals of 1 h, 2 h, 3 h, 4 h, 5 h, 6 h and 7 h during the second rest period at room temperature. Finally, the aliquots were diluted to 10^{-2} and 10^{-4} in sterile physiological saline solution. The direct and

diluted solutions were spread over NA and incubated at 37°C for 24 h. After incubation, the colonies on the agar surface were counted.

Voltage values during the electric current procedure were measured at each of the aforementioned intervals using VARIAC.

The $\log_{10}$ reduction factor (RF) for each treatment time was calculated according to the following formula: $RF = \log_{10} n_b - \log_{10} n_a$,
where n_b is the initial number of viable cells (CFU/ml) in the inoculum in the test medium, and n_a is the number of viable cells (CFU/ml) in the inoculum after treatment with 188 $\mu A/m^2$ DC and 251 $\mu A/m^2$ AC currents or/and the agent.

The electric current and agent treatments were applied at room temperature. Each test was thrice conducted. Similar bacterial numbers were counted in these experiments. The mean value of these three experiments was used. The pH and temperature of all samples were measured at each of the aforementioned intervals using a pH meter (Sartorius Professional Meter PP-50 AG, Goettingen, Germany).

3. Results and Discussions

The most common Gram-positive genera on the salted hides were *Staphylococcus* (115 isolates), *Bacillus* (111 isolates) and *Enterococcus* (75 isolates), while the most common Gram-negative genera on the salted hides were found to be *Enterobacter* (66 isolates), *Pseudomonas* (59 isolates) and *Vibrio* (32 isolates) (E. ASLAN & al. [6], E. ASLAN & al. [7]). Therefore, proteolytic *Vibrio fluvialis* and *Enterobacter cloacae,* proteolytic and lipolytic *Pseudomonas luteola, Enterococcus faecium*, *Staphylococcus cohnii* and *Bacillus pumilus* were selected as test isolates in this study.

Our earlier studies showed that the numbers of bacteria on salted hides were high due to an inadequately applied salt-pack curing process. In order to prevent bacterial damage on hides during brine curing process, the effect of DC treatment applied together with AC in a sequential manner and bronopol on the mixed culture of test isolates in the hide brine solution containing organic substance and 25% NaCl was examined in the present study. Three different experiments were conducted in this study.

Experiment 1 – was carried out to examine the effect of 1 g/L bronopol on the mixed culture during 10 h storage at room temperature. To prevent bacterial damage on the hides, bronopol was advised by researchers as an effective bactericide (L. MUTHUSUBRAMANIAN & al. [25]). In addition, bronopol has been used in several industrial area, from the leather industry to cosmetics. It has been stated that bronopol containing bactericides is fairly effective against different microorganisms and may be used in different applications (P.A. GEISS [26], D.M. BRYCE & al. [27]). Minimal inhibitory concentrations of bronopol against *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Candida albicans* and *Aspergillus niger* were found to be 62.5, 31.25, 31.25, 50 and 50 mg/ml, respectively. Bronopol forms disulfide bonds with thiol groups to denature proteins and it inhibits dehydrogenase activity (P.A. GEISS [26], D.M. BRYCE & al. [27]). It is used at 0.01% to 0.1% in cosmetics, pharmaceutical and household products.

Table 1. Values of temperature, bacterial cell counts and reduction factors of the mixed culture obtained from the experiment 1.

ET[a] with 1 g/L of Bronopol (h)	Temperature (°C)	Bacterial Cell Counts (CFU/ml)	Bacterial Cell Counts (Log_{10})	Reduction Factor (Log_{10})
BE[b]	20	2.77×10^7	7.44	-
W 1[c]	24	1.70×10^7	7.23	0.21
W 2	27	7.80×10^6	6.89	0.55
W 3	28	7.10×10^6	6.85	0.59
W 4	28	5.80×10^6	6.76	0.68
W 5	28	5.00×10^6	6.70	0.74
W 6	28	4.00×10^5	5.60	1.84
W 7	28	2.80×10^4	4.45	2.99
W 8	28	1.00×10^3	3.00	4.44
W 9	28	9.20×10^2	2.96	4.48
W 10	28	-	-	7.44

[a]Exposure time, [b]Before experiment, [c]Waiting hours

In the experiment 1, the bacterial cell counts of the mixed culture were reduced slowly (0.74 log_{10} RF) with bronopol after 5 h exposure time. Then, the bacterial cell counts were reduced rapidly with the test agent between 6 h and 9 h exposure times. At the end of the experiment, all of the bacteria in the mixed culture were killed with the antibacterial agent (Table 1). Researchers stated that bacterial cell numbers in soak liquors should be less than 10^5 (R. RANGARAJAN & al. [3]). In this experiment, the bacterial cell number of the mixed culture in the liquid test medium containing bronopol reduced to the recommended level at (2.80×10^4 CFU/ml) which damage of bacteria can be lowered after 7 h exposure time (Table 1).

It was stated that biofilms on medical devices cannot be annihilated by antimicrobials and antibiotics. Researchers found that weak electric current treatment increased the efficiency of antibiotics to exterminate biofilm on medical devices (A.E. KHOURY & al. [17]). The resistance of bacteria on salted hides against commonly used antibacterial agents may be similar to that of bacteria on medical devices (J.W. COSTERTON & al. [16], A.E. KHOURY & al. [17], S.A. BLENKINSOPP & al. [18], J. JASS & al. [19]). Hence, we wanted to examine herein whether or not killing of a mixed culture of hide bacteria by bronopol can be dramatically increased by the combined usage of direct electric current treatment applied together with alternating electric current. Therefore, *Experiment 2* was performed to determine the synergistic effect of DC treatment applied alternately with AC followed by 1 g/L of bronopol on the mixed culture of hide bacteria during 10 h storage at room temperature. The temperature of the test medium was 20°C before experiment. The temperature of the test medium increased slowly and reached 30°C after 20 min.

Table 2. Values of temperature, voltage, bacterial cell counts, reduction factors of the mixed culture obtained from experiment 2.

ET with Electric Current (min)	Temperature (^oC)	Voltage	Bacterial Cell Counts (CFU/ml)	acterial Cell ounts (Log_{10})	Reduction Factor (Log_{10})
BE	20		1.77×10^7	7.25	
2 DC	21	5.8	1.30×10^7	7.11	0.14
4 AC	22	5.8	1.10×10^7	7.04	0.21
6 DC	23	5.9	6.50×10^6	6.81	0.44
8 AC	24	5.9	4.00×10^6	6.60	0.65
10 DC	25	5.6	1.00×10^6	6.00	1.25
12 AC	26	5.6	1.70×10^5	5.23	2.02
14 DC	27	5.3	1.25×10^5	5.10	2.15
16 AC	28	5.3	1.05×10^5	5.02	2.23
18 DC	29	5.2	7.00×10^4	4.85	2.40
20 AC	30	5.2	4.30×10^4	4.63	2.62
ET with 1 g/L of Bronopol (h)					
W 1	30		6.00×10^3	3.78	3.47
W 2	30		1.00×10^3	3.00	4.25
W 3	29		7.40×10^2	2.87	4.38
W 4	29		4.40×10^2	2.64	4.61
W 5	28		2.30×10^2	2.36	4.89
W 6	28		1.10×10^2	2.04	5.21
W 7	28		1.20×10^1	1.08	6.17
W 8	28		-	-	7.25
W 9	28		-	-	7.25
W10	28		-	-	7.25

In this experiment, the bacterial cell numbers reduced slightly during the electric treatment for 20 min. The bacterial cell number of the mixed culture reduced from 1.77×10^7 CFU/ml to 7.00×10^4 CFU/ml (2.40 $\log_{10}$ RF) after the electric treatment for 18 min. Although 0.85 $\log_{10}$ RF of the bacteria damaged by the electric current was detected after 1 h exposure time with 1 g/L of the test agent in experiment 2, 0.21 $\log_{10}$ RF of the mixed culture was detected after 1 h exposure time with the test agent in experiment 1. The bacteria damaged by the electric current were killed in a shorter time than that required for their inactivation only by bronopol. While the mixed culture of bacteria damaged by the electric current was completely inactivated with 1 g/L of bronopol after 8 h contact time, the mixed culture was completely inactivated after 10 h exposure time with only bronopol (Tables 1 and 2). The bacteria damaged by the electric current were reduced to the recommended level (< 10^5 CFU/ml) in a shorter time than was required for their reduction using only bronopol. Although the bacterial cell number was reduced to 7.00×10^4 CFU/ml (2.40 $\log_{10}$ RF) by the electric current after only 18 min, 7 h treatment with the test agent was necessary for reduction of the test bacteria to 2.80×10^4 CFU/ml (2.99 $\log_{10}$ RF) in the experiment 1 (Tables 1 and 2).

Comparable research results also were recorded from the other study. Investigators observed that biofilm of *Pseudomonas aeruginosa* was not killed by the application of an electric current alone, and that biofilm bacteria on electrically passive inserts within electric fields were killed by low concentrations of an antibiotic. In that study it was observed that the penetration of antibiotic into the biofilm was enhanced by an electric field (J.W. COSTERTON & al. [16]).

In our previous study it was found that 15 min exposure to 188 μA/m^2 AC destroyed Gram-negative bacteria (*Enterobacter cloacae, Pseudomonas luteola* and *Vibrio fluvialis*) in the brine solution. Additionally, 188 μA/m^2 AC for 15 min, followed by 188 μA/m^2 DC for one min, destroyed the mixed culture of these bacteria in the brine solution (Y. BIRBIR & al. [21]).

Experiment 3 was conducted to detect the effect of 188 μA/m^2 DC treatment applied together with 251 μA/m^2 AC in a sequential manner and 1 g/L of bronopol on the mixed culture of hide bacteria. In this experiment, the electric treatments were applied to the mixed culture of bacteria in two separate cycles before application of bronopol. In the first cycle, 20 min of 188 μA/m^2 DC treatment sequentially paired with 251 μA/m^2 AC was applied to the mixed culture of hide bacteria in the liquid medium. Then, this medium was left alone for 3 h at room temperature. After 3 h, a total of 12 min of 188 μA/m^2 DC treatment sequentially paired with 251 μA/m^2 AC was applied to the mixed culture of hide bacteria in the liquid medium. Later, 1 g/L of bronopol was added and the test medium, and this medium was left for 7 h at room temperature. At the end of the first electric treatment cycle, temperature of the test medium reached 30°C, and at the end of the second electric treatment cycle it reached 35°C (Table 3).

Table 3. Values of temperature, voltage, bacterial cell counts, reduction factors of the mixed culture obtained from experiment 3.

ET with Electric Current (min)	Temperature (°C)	Voltage	Bacterial Cell Counts (CFU/ml)	Bacterial Cell Counts (Log_{10})	Reduction Factor (Log_{10})
BE	20	-	4.56×10^7	7.66	
2 DC	21	6.6	3.06×10^7	7.49	0.17
4 AC	22	7.1	2.80×10^7	7.45	0.21
6 DC	23	6.2	1.50×10^7	7.18	0.48
8 AC	24	6.4	1.00×10^7	7.00	0.66
10 DC	25	5.9	4.00×10^6	6.60	1.06
12 AC	26	6.1	4.50×10^5	5.65	2.01
14 DC	27	5.3	3.80×10^5	5.58	2.08
16 AC	28	5.7	2.90×10^5	5.46	2.20
18 DC	29	5.4	1.59×10^5	5.20	2.46
20 AC	30	5.5	1.15×10^5	5.06	2.60
Storage Duration (h)					
W 1	30	-	1.20×10^4	4.08	3.58
W 2	30	-	1.10×10^4	4.04	3.62
W 3	29	-	1.00×10^4	4.00	3.66
ET (min)					
2 DC	30	6.3	6.00×10^3	3.78	3.88
4 AC	31	6.6	5.00×10^3	3.70	3.96
6 DC	32	6.1	8.40×10^2	2.92	4.74
8 AC	33	6.3	7.30×10^2	2.86	4.80
10 DC	34	5.9	3.00×10^2	2.48	5.18
12 AC	35	6.2	1.20×10^2	2.08	5.58
ET with 1 g/L of Bronopol (h)					
W 1	33	-	1.00×10^2	2.00	5.66
W 2	32	-	5.00×10^1	1.70	5.96
W 3	31	-	-	-	7.66
W 4	30	-	-	-	7.66
W 5	29	-	-	-	7.66
W 6	28	-	-	-	7.66
W 7	28	-	-	-	7.66

The bacterial cell count was reduced from 4.56×10^7 CFU/ml to 1.15×10^5 CFU/ml (2.60 $\log_{10}$ RF) after the treatment of DC applied sequentially with AC for 20 min. After the electric treatment, the bacterial cell counts decreased during 3 h storage at room temperature, but the reduction of the bacterial cell counts (<1 $\log_{10}$ RF) was notably low. After the application of second DC/AC treatment cycle, the bacterial cell count was reduced from 1.00×10^4 to 1.20×10^2 CFU/ml (totaly 5.58 $\log_{10}$ RF). These results were similar to those reported by other studies (J.L. DEL POZO & al. [28]). In this research, the long-term pre-effect of 20, 200 and 2000 μA DC on *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Staphylococcus epidermidis* biofilms (>5 $\log_{10}$ CFU/cm^2) was investigated by researchers. A time-dependent reduction in biofilm viability was seen, with usually a lower viable cell counts detected when electrical current was utilized for longer periods of time. When the biofilms of *Staphylococcus aureus* and *Staphylococcus epidermidis* were treated with 2000 μA DC for 2 days, 5 to 6 $\log_{10}$ reduction in viable cell counts was detected. In addition, 3.5 to 5 $\log_{10}$ RFwas detected when biofilms of *Pseudomonas aeruginosa* were treated with 2000 μA for seven days (J.L. DEL POZO & al. [28]). In our experiment, the mixed culture was completely inactivated after the treatment with 1 g/L of bronopol for 3 h and $\log_{10}$ RF of the mixed culture was 7.66. After two cycles of electric current, the mixed culture of bacteria was killed in a shorter time than the time required for their inactivation by only bronopol (Table 3). The electric current treatment applied in this study could have increased transport of bronopol into the damaged bacterial cells. Although inactivation effect of electric current on bacteria was not known exactly, it is thought that the electric current may damage bacterial membrane. It may also affect cell wall, the orientation of membrane lipids and, consequently, cell viability. In addition, different chemical oxidants such as hydrogen peroxide, ozone, free chlorine and chlorine dioxide formed during the electric treatment may damage bacterial cells (J.C. PARK & al. [23], D.S. DIMITROV & al. [29], K.P. DREES & al. [30], J.S. CHANG & al. [31], C.P. DAVIS & al. [32]).

4. Conclusions

This investigation evaluates the effect of a combined electric current treatment using both DC and AC in a sequential manner, followed by bronopol treatment on the mixed culture of hide bacteria in the liquid medium consisting of organic matters and high concentration of NaCl. In the present study, after 14-20 min treatment with 251 μA/m^2 AC applied in sequence with 188 μA/m^2 DC, the bacterial cell count of the mixed culture fell to the reasonable level at which the bacteria cannot damage the hide. However, to obtain the same level results (10^4 CFU/ml), straight bronopol required 7 h exposure time. Combined application of the electric currents and bronopol to inactivate the hide bacteria at room temperature yielded a substantial inactivation accompanied by a complete eradication of the mix culture. This study showed that the hide bacteria damaged via two cycles of DC treatment applied in sequence with AC can subsequently be killed easily by antibacterial agents. In conclusion, combinatory usage of DC and AC and antibacterial agent may be applied in hide-brine curing liquors to prevent proteolytic and lipolytic bacterial activities on hides.

5. Acknowledgements

We are grateful to the tanneries located in Tuzla-Istanbul Leather Organized Tannery Region in Turkey and Buckman International for providing us the hide samples and bronopol, respectively. We also thank Mr. Elton Hurlow and Dr. Martin Louis Duncan for their valuable advice for this manuscript.

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