Synergistic Effect of Direct and Alternating Electric Current Treatments and Bronopol to Inactivate Bacteria Found in Hide Soak Liquors

by

Y. Birbir,[1] N. Dolek,[2] M. Birbir[3]* and P. Caglayan[3]

[1]*Marmara University, Faculty of Technology, Department of Electric and Electronic Engineering,*

[2]*Marmara University, Institute for Graduate Studies in Pure and Applied Science,*

[3]*Marmara University, Faculty of Science and Letters, Department of Biology,*

34722 Goztepe, Istanbul, Turkey

Abstract

It is well known that bacteria on hides and in the hide-soak liquors do damage that compromises leather quality. These harmful bacteria are resistant to the antibacterial agents used in the hide industry. In this research the synergistic effect of a combined electric current treatment using both 1.5 A direct and 2.0 A alternating electric currents, followed by 1 g/L of bronopol treatment on mixed culture of Gram positive and Gram negative hide bacteria was examined in a liquid medium containing 2% NaCl and organic substances. *Enterobacter cloacae*, *Vibrio fluvialis*, *Pseudomonas luteola*, *Staphylococcus cohnii*, *Enterococcus faecium* and *Bacillus pumilus* were used as test isolates. Six different experiments were carried out on the mixed culture of test bacteria to determine the efficiency of the synergistic effect of a combined electric current treatment. Bacterial cell counts were reduced to low level in a short period using the electric treatment. More than 2 log reduction factor of the mix culture was observed within 12 min after the electric current treatment, and the damaged bacteria were killed easily by bronopol in five hours. DC and AC electric currents in combination with antimicrobial agents may be used to more efficiently exterminate the bacteria found in the hide-soak liquors.

Resumen

Es bien conocido que las bacterias en las pieles y en los licores de remojo provocan daños que comprometen la calidad del cuero. Estas bacterias dañinas son resistentes a los agentes antibacterianos utilizados en la industria de la piel. En esta investigación el efecto sinérgico de un tratamiento combinado de corriente eléctrica utilizando ambas corrientes eléctricas, 1.5A contínua y 2,0A alterna, seguido de 1 g/l de tratamiento con bronopol en cultivo mixto de bacterias Gram positivas y Gram negativas se examinó en un medio líquido que contiene 2% de NaCl y sustancias orgánicas. Enterobacter cloacae, Vibrio fluvialis, Pseudomonas luteola, Staphylococcus cohnii, Enterococcus faecium y Bacillus pumilus se utilizaron como cepas de prueba. Seis diferentes experimentos se llevaron a cabo en el cultivo mixto de bacterias de prueba para determinar la eficiencia del efecto sinérgico de un tratamiento combinado de corriente eléctrica. Los recuentos de células bacterianas fueron reducidos a un nivel bajo en un corto período de tiempo con el tratamiento eléctrico. Se observó más de 2 factores de reducción logarítmica de la cultura de la mezcla dentro de los 12 minutos posteriores al tratamiento con corriente eléctrica, y las bacterias dañadas murieron fácilmente por bronopol en cinco horas. Corrientes eléctricas contínuas y alternas en combinación con agentes antimicrobianos pueden ser utilizados de manera más eficiente para exterminar las bacterias que se encuentran en los licores de remojo.

*Corresponding author e-mail: mbirbir@marmara.edu.tr

Manuscript received April 27, 2013, accepted for publication July 12, 2013.

INTRODUCTION

Antimicrobial applications are commonly used in hide preservation and main-soak liquors to prevent bacterial damage during storage of hides and soaking processes. Hide preservation, which controls bacterial activities on hide, is a temporary preservation method until a hide is processed into a final product.[1] In some countries; fresh hides are cured by salt and boric acid. After the curing process, the hides can be stored for long periods and sent to tanneries to make leather. The first process in the tannery is soaking process. This process removes excess salt, blood, soluble proteins, dirt and manure from hides. Besides, this process softens up and rehydrates the partially dehydrated cured hide to restore it to its original condition of 65% water. Soaking processes are commonly applied in two stages. The pre-soaking process is applied for three or four hours to remove excess salt from the hides. The duration of the main-soaking process may change according to the type and condition of the cured stock, physical conditions of the raw stock and fluctuations in the beamhouse operations. This duration may range from about 1.5 hours to 24 hours in different countries. The duration of soaking process is suggested as eight hours or more. Most tanneries usually prefer a 24-hours soaking process for adequate rehydration of hides.[2-4] To prevent bacterial damage on the hides during long soaking process, 0.1-0.3% antibacterial agents have been suggested for hide soaking process.

Although antibacterial agents have been used in preservation and soaking processes of the hides, high bacterial populations were isolated from the salted and soaked hides in our previous studies.[5-7] These studies proved that inactivation of bacteria on the salted hides and soaked hides was influenced by many factors, including effect of antimicrobial agents on growth (bacteriocidal or bacteriostatic), their selective toxicity, resistance against antibacterial agents commonly used in the leather industry, the concentration of the agent, the exposure time to the agent, the temperature of the medium, the type of process, the chemical composition of the medium and the pH of the medium.[8,9] Moreover, chemicals such as salt and boric acid used in the hide preservation have bacteriostatic effect.[10-12] In addition, the bacteria can develop resistance against antibacterial agents and transfer this resistance gene, which is located on plasmid, to the other bacteria that live together on the hides. Therefore, the bacteria on salted hides cannot be completely killed, and these bacteria find an ideal medium to grow at an enormous rate in the soak liquors and on the soaked hides. It was observed that the concentrations of antibacterial agent necessary to inactivate bacteria in hide-soak liquor were often much higher than those recommended by the chemical companies or the concentrations applied in the leather industries.[12,13] As detected in our previous results, using only antimicrobial agents to inactivate bacteria found in soak liquors, salted and soaked hides cannot easily prevent bacterial activities in the leather industry.[5-7, 13-15] Since a similar observation was detected by Dr. David G. Bailey, electron beam irridation was applied in the leather industry in order to prevent bacterial growth on hides.[16, 17]

In recent years, we researched the effect of electric current to prevent bacterial growth on hides.[5, 18-19] In these studies, inactivation effect of direct electric current against microorganisms found in salt, the pre-soaking and the main-soaking was examined.[5, 18-19] A total of 14 soak liquors containing the pre-soak liquors (Sample 1-7) and the main-soak liquors (Samples 8-14) were obtained from different tanneries in Turkey. The bacterial numbers in the main-soak liquors ($5.0x10^3$-$2.0x10^7$ c.f.u./mL) were higher than that of the pre-soak liquors ($6.5x10^4$-$8.0x10^5$ c.f.u./mL). These main-soak liquors had been treated with different antibacterial agents, but the bacterial growth numbers in these soak liquors were still high.[5] Although all non-halophilic bacteria in the pre-soak liquors were inactivated via 2 A direct electric current within 20-30 min, inactivation of the bacteria in the main-soak liquors occurred within 50 min.[5] In the study of salt, proteolytic and lipolytic extremely halophilic archaea isolated from salt were inactivated via 0.5A low level direct electric current in liquid media containing organic substances and 25% NaCl. Although inactivation time varied according to the species of extremely halophilic archaea, the electric current killed all species tested within 20 min.[18] In addition, 0.1 A, 0.2 A, 0.3 A and 0.4 A direct electric current inactivated extremely halophilic archaea in salt, which was dissolved in the brine solution, within 15 min, 10 min, 5 min and 3 min, respectively. Within 1 min, 0.5 A direct electric current inactivated extremely halophilic archaea in the salt samples (10^4-10^5c.f.u./g) which were dissolved in the brine solution, while treatment with 0.5 A direct electric current for 15 min was necessary to inactivate the extremely halophilic archaea in the salt samples dissolved in the liquid Brown media. Also, within 10 min, 0.5 A direct electric current inactivated lipase and protease producing extremely halophilic archaea (10^5-10^6 c.f.u./mL), which were grown separately in the liquid Brown media.[19]

In the leather industry, a wide variety of antibacterial agents containing different active ingredients have been developed by scientists to combat bacterial resistance. The most common classes of antibacterial agents used in the leather industry are the dithiocarbamates and the isothiazolinones. Potassium dimethyl-dithiocarbamates, organosulfur compound, phenol and 4-chloro-3-methyl, and quaternary ammonium compound composed of 12.5% didecyl dimethyl ammonium chloride and 12.5% benzyl dimethyl ammonium chloride are among the antibacterial agents used in the Turkish Leather Industry. Bronopol tested in the present study was advised as an effective antibacterial agent by researchers to prevent bacterial damage occurring in the main-soak liquors.[20] Bronopol can be synthesized by reacting bromo-nitro-methyl-cyclohexanol and formaldehyde[21, 22] or by reacting nitroalkane with formaldehyde.[20] The active ingredient of bronopol is 2-bromo-

2-nitropropane-1,3-diol. It is white crystalline powder and does not have any odor. It shows bactericidal effect against Gram positive and Gram negative bacteria. Bronopol is used to control microorganisms found in pharmaceutical products, toiletries, consumer products, cooling towers, water treatment, metal working fluids, fuel/oil storage tanks, water-based paints, inks and adhesives.[23,24]

As we know, bacteria try to survive in the presence of antibacterial agents applied in industries by protecting themselves with different mechanisms.[8] Finally they can develop resistance to the applied antibacterial agents. Therefore, researchers have developed new strategies to solve this problem. The synergistic effect is among these new strategies. The importance of synergy has been realized in several sectors.[9, 23] The synergistic effect of two or more antimicrobial treatments has been studied by investigators in different fields. Previous studies indicated that antimicrobial agents and electric current acted synergistically to inactivate microorganisms.[25, 26]

The synergistic effect of gamma radiation and antibacterial agent was examined in the leather industry by Dr. David G. Bailey in 1975. In the researcher's study, cattle hides were preserved with a combination of gamma radiation and bactericide. In that study, the synergistic effect of antibacterial agent and gamma radiation was used to control the destructive effect of bacteria on the hides. The researcher explained that the hides treated with 10KGys of gamma radiation and 0.3% antibacterial agent containing 1,2 benzisothiazolin-3-one did not contain bacteria nor give off an odor that shows putrefaction of the hides. In addition, the hairslip was not detected on these hides.[16] Similar results were obtained from the hides preserved with 0.03% of the same antibacterial agent and 30KGys of gamma radiation. In the other study, electron beam irradiation was applied to fresh-fleshed hides treated with 0.3% of the same antibacterial agent in a drum. Bacterial count on the hides was reduced to a low level by the synergistic effect of antibacterial agent and electron beam irradiation. The researcher emphasized that this method can be used successfully for preservation of hides without salt to yield commercial grade leather.[17]

Electric current applications have been investigated to prevent bacterial and archaeal growth in different industries including hide industry. Furthermore, it is used to prevent biofilm formation and to enhance the activity of antibacterials against established biofilms.[25] Despite the positive results in the prior work cited, the synergistic effect of a combined electric current treatment using both direct and alternating electric currents, followed by bronopol treatment on mixed culture of Gram positive bacteria (*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 liquid medium containing organic substance and 2% NaCl has not heretofore been reported. The goal of the present study was to determine the synergistic effect of direct electric current treatment applied together with alternating electric current and bronopol. Therefore, we designed the experiment to determine the inactivation effect of the test agent; inactivation effect of direct electric current (DC) treatment applied together with alternating electric current (AC); the combined usage of DC treatment applied together with AC and bronopol on the mixed culture of bacteria in the hide-soak liquor. Thus, we wanted to determine the complete inactivation time of the test bacteria damaged by the electric current treatment and subsequent bronopol.

EXPERIMENTAL

Test Bacteria

Gram negative (*Enterobacter cloacae, Vibrio fluvialis* and *Pseudomonas luteola*), Gram positive (*Staphylococcus cohnii* and *Enterococcus faecium*) and Gram positive endospor forming bacteria (*Bacillus pumilus*) were selected as the test bacteria. *Pseudomonas luteola, Enterococcus faecium, Staphylococcus cohnii* and Bacillus *pumilus* showed both proteolytic and lipolytic activities, while *Enterobacter cloacae* and *Vibrio fluvialis* were found as protease positive and lipase negative in our previous study.[27] These isolates were the most commonly found bacterial species on the salt-pack cured hides.[14,15] The test isolates were identified by API test kits (BioMérieux, Inc, France) in the previous studies.[14,15] API® 20E (bioMériux, Inc, France), API® 20NE (bioMériux, Inc, France), API Staph (bioMériux, Inc, France), API 20 Strep (bioMériux, Inc, France) and API 50CH (bioMériux, Inc, France) were used for the identification of *Enterobacter cloacae* and *Vibrio fluvialis,* P*seudomonas luteola, Staphylococcus cohnii*, *Enterococcus faecium* and *Bacillus pumilus,* respectively.

The Antibacterial Agent

The agent containing 2-Bromo-2-nitropropane-1,3-diol as an active ingredient was used as a test bactericide. The agent concentration used in the present study was 1 g/L. Bronopol was obtained from Buckman International, Memphis, TN.

Preparation of the Mixed Culture

Enterobacter cloacae, Vibrio fluvialis, Pseudomonas luteola, Staphylococcus cohnii, Enterococcus faecium and *Bacillus pumilus* were separately grown in Nutrient Broth (Merck, Darmstadt, Germany) at 37°C for 24 hours. After the incubation period, each of these bacterial cultures was separately suspended in sterilized physiological saline solution to a final cell density of 10^8 c.f.u./mL. Then, the mixed culture of the test isolates was prepared from these physiological saline solutions. Thirty mL of each bacterial culture was used in the mixture.

The Electrolysis Cell System

20 mL of the bacterial suspension was transferred into an electrolysis cell containing 180 mL of Nutrient Broth (NB) and 2% NaCl. A 100 mL quantity of the test medium was removed from the electrolysis cell before the experiments and diluted to 10^{-4} with sterilized physiological saline solution. Then, the diluted solution was spread over Nutrient Agar (NA) (Merck, Darmstadt, Germany) and incubated at 37°C for 24 hours. After the incubation period, the colonies on the agar surface were counted. This medium was used in all of the experiments accomplished in the present study. The electrolysis cell used was made of a glass beaker having two internally attached platinum wire electrodes immersed in the test medium. The two electrodes were 1 mm in diameter and 80 mm in length, at a separation of 40 mm. They were connected to a variable alternating current source (VARIAC), (Input= 220V, f = 50 Hz, VA = 2250 VA), with AC variable output voltage range of 0–220 V and alternating power output electric current level 0–10A. There is an AC-DC main switch for AC and DC output power supply selection, DC power output level with variable output voltage range of 0–200V and current range of 0–10A. Voltage values during the electric current treatment were measured at each of the above-mentioned intervals using VARIAC.[18,28]

Six Different Experiments were Carried out in this Study

Experiment 1; The test medium containing the mixed culture, NB, and 2% NaCl was used in experiment 1. This medium was left at room temperature for eight hours. Then, aliquots of 100 μl were removed from the test medium at intervals of 1h, 2h, 3h, 4h, 5h, 6h, 7h and 8h and diluted to 10^{-2}, 10^{-4} and 10^{-6} in sterilized physiological saline solution. Then, the direct and diluted solutions were spread over Nutrient agar and incubated at 37°C for 24 hours. After the incubation period, the colonies on the agar surface were counted.

The $\log_{10}$-increase factor (IF) for this experiment was calculated according to the following formula:

$$IF=\log_{10} n_a-\log_{10} n_b$$

where n_a is the count of viable bacterial cells (c.f.u./mL) in the inoculum during storage, and n_b is the initial count of viable bacterial cells (c.f.u./mL) in the inoculum in the test medium.

Experiment 2; The test medium containing the mixed culture, NB, 2% NaCl and 1 g/L of the test agent was used in experiment 2. The bacteria in the test medium were treated with the test agent for eight hours at room temperature. Then, 100 μl of the treated bacterial suspensions were removed from the test medium at intervals of 1h, 2h, 3h, 4h, 5h, 6h, 7h and 8h during the experiment and these aliquots were diluted to 10^{-2} and 10^{-4} in the sterilized physiological saline solution. Later, the direct and diluted solutions were spread over Nutrient agar and incubated at 37°C for 24 hours. After the incubation period, the colonies on the agar surface were counted.

Experiment 3; The test medium containing the mixed culture, NB and 2% NaCl was used in experiment 3. Firstly, 1.5 A DC was applied for two min and followed by 2 A AC treatment for two more min. The test medium was left standing for four min after applications of DC and AC treatments. Then, this electric treatment was repeated three times (total of 12 min of DC and AC treatments and eight min without any treatment) until the temperature of the medium reached 26°C. Afterwards, 1 g/L of the test agent was added into the medium and this medium was left at room temperature for eight hours. Aliquots of 100 μl were removed from this medium at intervals of 2, 4, 6, 8, 10 and 12 min during DC and AC treatments and subsequently at intervals of 1h, 2h, 3h, 4h, 5h, 6h, 7h and 8h after the test agent was added into the test medium. Then, these aliquots were diluted to 10^{-2} and 10^{-4} in the sterilized physiological saline solution, and the direct and diluted solutions were spread over NA and incubated at 37°C for 24 hours. After the incubation period, the colonies on the agar surface were counted.

Experiment 4; The test medium containing the mixed culture, NB and 2% NaCl was used in experiment 4. Firstly, 1.5 A DC was applied for two min and followed by 2 A AC treatment for two more min. The test medium was left standing for four min after application of DC and AC treatments. Then, this electric treatment was repeated three times (total of 12 min of DC and AC treatments and eight min without any treatment) until the temperature of the medium reached 26°C. Afterwards, the treated medium was left at room temperature for 8 hours without any treatment. Aliquots of 100 μl were removed from this medium at intervals of 2, 4, 6, 8, 10 and 12 min during DC and AC treatments and subsequently at intervals of 1h, 2h, 3h, 4h, 5h, 6h, 7h and 8h. These aliquots were diluted to 10^{-2} and 10^{-4} in the sterilized physiological saline solution, and both direct and diluted solutions were spread over NA and incubated at 37°C for 24 hours. After the incubation period, the colonies on the agar surface were counted.

Experiment 5; The test medium containing the mixed culture, NB and 2% NaCl was used in experiment 5. Firstly, 1.5 A DC was applied for two min and followed by 2 A AC treatment for two more min. The test medium was left standing for four min after application of DC and AC treatments. Then, this electric treatment was repeated three times (total of 12 min of DC and AC treatments and eight min without any treatment) until the temperature of the medium reached 26°C. Afterwards, the medium was left at room temperature for three hours without any treatment. Then, 1.5 A DC was applied for two min and followed by 2 A AC treatment for two more min. Again, the test medium was left standing for four min after applications of DC and AC treatments. This electric treatment was repeated two times (total of eight min of DC and AC treatments and four min without any treatments) until the temperature of the medium reached 30°C and left standing for five hours at room temperature without any treatments. Aliquots of 100 μl were removed from this medium at intervals of 2, 4, 6, 8, 10 and 12

min during the first DC and AC treatments; at intervals of 1h, 2h, 3h during the first waiting hours at room temperature; at intervals of 2, 4, 6 and 8 min during the second DC and AC treatments; at intervals of 1h, 2h, 3h, 4h, 5h during the second waiting period at room temperature. Then, the aliquots were diluted to 10^{-2} and 10^{-4} in sterilized physiological saline solution. Later, the direct and diluted solutions were spread over NA and incubated at 37°C for 24 hours. After the incubation period, the colonies on the agar surface were counted.

Experiment 6; The test medium containing the mixed culture, NB and 2% NaCl was used in experiment 6. Firstly, 1.5 A DC was applied for two min and followed by 2 A AC treatment for two more min. The test medium was left standing for four min after application of DC and AC treatments. This electric treatment was repeated three times (total of 12 min of DC and AC treatments and eight min without any treatment) until the temperature of the medium reached 26°C. Afterwards, the medium was left standing for three hours at room temperature without any treatment. Then, 1 g/L of the test agent was added and left standing for five hours without any treatments at room temperature (total of eight hours and 20 minutes). Aliquots of 100 μl were removed from this medium at intervals of 2, 4, 6, 8, 10 and 12 min during the DC and AC treatments; at intervals of 1h, 2h, 3h during the first waiting hours; at intervals of 2, 4, 6 and 8 min during the second DC and AC treatments; at intervals of 4h, 5h, 6h, 7h and 8h after the antibacterial agent was added. Then, the aliquots were diluted to 10^{-2} and 10^{-4} in sterilized physiological saline solution. The direct and diluted solutions were spread over NA and incubated at 37°C for 24 hours. After the incubation period, the colonies on the agar surface were counted.

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 (c.f.u./mL) in the inoculum in the test medium, and n_a is the number of viable cells (c.f.u./mL) in the inoculum after treatment with 1.5 A DC and 2 A AC currents or the agent.

The pH values and temperatures of the test media were 7 and 20°C before treatment, respectively. The electric current and agent treatments were conducted at room temperature. Each test was repeated three times using 200 mL of the test media. Similar bacterial counts were obtained from these experiments. The mean value of these three experiments was used. The pH and temperature of all samples were measured at each of the above-mentioned intervals using a pH meter (Sartorius Professional Meter PP-50 AG, Goettingen, Germany).

Results and Discussion

It is well known that the bacteria on the hides and in the hide-soak liquors cannot be inactivated easily by the antibacterial agents used in the hide industry.[6,7] Data obtained from our previous experiments proved that this problem is common in the leather industry. As known, all of the bacteria cannot be killed by antibacterial agents; subsequently some of these bacteria can develop antibacterial resistance against the commonly used antibacterial agents. These bacteria can transfer resistance to sensitive bacteria living in the same environment, and finally resistance genes become common among the bacteria in the same environment. Transfer of resistance genes, which were developed against antibacterial agent, has been usually accomplished via plasmid. Some properties of bacteria such as antibiotic production, conjugation, decomposition of different toxic substances including naphthalene, utilization of diverse carbon sources, resistance against different antibiotics and toxic metals, animal cell invasion, productions of toxin, bacteriocin, and capsule were contributed by plasmids.[8] In our previous studies, although the antibacterial agents were used for prevention of bacteria in the soak liquors, the bacterial cell counts on the 34 soaked hides were high (10^5-10^8 c.f.u./g). Proteolytic and lipolytic bacterial cell counts in these soaked hides were between 10^4 and 10^8 c.f.u./g.[6] In another study, 10^3-10^7 c.f.u./mL of bacteria were isolated from seven of the main-soak liqours treated with different antibacterial agents.[5] Our previous studies verified that presence of high bacterial cells in the hide soak liquors and on the soaked hides were related with poor preservation methods. Therefore, to overcome this problem, we employed electric current together with bronopol to inactivate hide bacteria before destruction of the hides occurred. Six different experiments were performed in this study.

Experiment 1 was performed to determine the increase in bacterial cells of the mixed culture in the liquid media containing organic substances and 2% NaCl such as soak liquor without any treatment during eight hours storage at room temperature. Temperature of test medium reached 24°C at the end of experiment (Table I).

As shown in Table I, the bacterial cell counts in the test medium gradually increased during the storage. While the bacterial cell count of the mixed culture in the test medium increased 1.45 log in two hours at room temperature, almost 2 log increase factor was detected at the end of experiment. These results showed that if bacterial populations in hide soak liquors cannot be inactivated by effective antibacterial agents, bacterial populations will increase rapidly and, consequently, decrease the leather's value. Therefore, the bacterial activity in soak liquors should be immediately prevented with effective antibacterial treatment (Table I).

TABLE I
Values of temperature, bacterial cell counts and increase factors of the mixed culture obtained from experiment 1.

Storage duration (hour)	Temperature (°C)	Bacterial cell counts (c.f.u./mL)	Increase factor (Log_{10})
BE[a]	20	$2.00x10^7$	7.30[b]
1	23	$5.00x10^7$	0.40
2	24	$5.60x10^8$	1.45
3	24	$7.20x10^8$	1.56
4	24	$8.30x10^8$	1.62
5	24	$9.20x10^8$	1.66
6	24	$1.36x10^9$	1.83
7	24	$1.42x10^9$	1.85
8	24	$1.87x10^9$	1.97

[a]BE: before experiment
[b]Log_{10} values of the mixed culture before storage at room temperature.

TABLE II
Values of temperature, bacterial cell counts and reduction factors of the mixed culture obtained from the experiment 2.

Treatment time with 1 g/L of bronopol (hour)	Temperature (°C)	Bacterial cell counts (c.f.u./mL)	Reduction factor (Log_{10})
BE[a]	20	$2.00x10^7$	7.30[b]
1	23	$1.20x10^7$	0.22
2	24	$6.70x10^6$	0.47
3	24	$5.10x10^5$	1.59
4	24	$6.70x10^4$	2.47
5	24	$8.00x10^3$	3.40
6	24	$7.40x10^2$	4.43
7	24	-	7.30
8	24	-	7.30

[a]BE: before experiment
[b]Log_{10} values of the mixed culture before the treatment with bronopol.

Experiment 2 was carried out to examine the effect of 1g/L bronopol on the mixed culture during eight hours storage at room temperature. Temperature of test medium reached 24°C at the end of experiment (Table II).

The mixed culture in the test medium was reduced > 2-4 log after four and six hours treatment with the test agent, respectively. The bacterial cell count of the mixed culture was reduced to $6.7x10^4$ c.f.u./mL (2.47 log RF) after four hours exposure time with bronopol. Researchers emphasized that bacterial cell count in soak liquors should be less than 10^5 c.f.u./mL.[3] Hence, this number was the reasonable level to which bacterial damage was reduced on the hides in soak liquors. Finally, the bacteria were completely inactivated with 1 g/L of the test agent at seven hours exposure time (Table II).

Experiment 3 was accomplished to determine the synergistic effect of DC treatment applied together with AC and bronopol on the mixed culture of hide bacteria during eight hours 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 26°C after 12 min and at the end of experiment was 25°C (Table III).

The bacterial cell count was reduced to $9.5x10^4$ c.f.u./mL (2.32 log RF) after 1.5 A DC treatment applied together with 2 A AC for 12 min. Then, 1 g/L of bronopol was added and the bacteria in the medium were treated with the antibacterial agent for eight hours. The bacteria damaged by the electric current were completely inactivated with 1 g/L of bronopol after five hours exposure time.

When the mixed bacterial culture, which was damaged by the electric current in the test medium, was treated with 1g/L of the test agent, 1.88 log RF (total of 4.20 log RF) of the mixed culture was detected after one hour exposure time. The mixed culture was completely inactivated after five hours exposure time with bronopol. The bacteria damaged by the electric current were killed in a shorter time than the time required for their inactivation by only bronopol. Although the bacterial cell count was reduced to $9.5x10^4$ c.f.u./mL (2.32 log RF) by the electric current for only 12 min, four hours treatment with the test agent was necessary for reduction of the test bacteria to $6.7x10^4$ c.f.u./mL (2.47 log RF) in experiment 2 (Tables II and III).

The main reason to do experiment 4 was to examine only the effect of 1.5 A DC treatment applied together with 2 A AC on

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

Treatment time with electric current (min)	Temperature (°C)	Voltage (V)	Bacterial cell counts (c.f.u./mL)	Reduction factor (Log_{10})
BE	20	-	$2.00x10^7$	7.30
2 DC	21	18.3	$1.43x10^7$	0.15
4 AC	22	26.9	$1.35x10^7$	0.17
6 DC	23	15.2	$3.20x10^6$	0.80
8 AC	24	22.1	$1.90x10^6$	1.02
10 DC	25	14.3	$1.89x10^5$	2.02
12 AC	26	20.1	$9.50x10^4$	2.32
Treatment time with 1 g/L of Bronopol (hour)				
1	26	-	$1.25x10^3$	4.20
2	26	-	$2.10x10^2$	4.98
3	25	-	$7.00x10^1$	5.46
4	25	-	$2.00x10^1$	6.00
5	25	-	-	7.30
6	25	-	-	7.30
7	25	-	-	7.30
8	25	-	-	7.30

the mixed culture of hide bacteria during and after application of the electric treatment. The electric current was applied for 12 min and then the bacteria in the liquid medium was left for eight hours at room temperature. Temperature of the test medium was 26°C after 12 min and at the end of experiment was 25°C (Table IV).

The bacterial cell count was reduced to $9.6x10^4$ c.f.u./mL (2.32 log RF) after the treatment of 1.5 A DC applied together with 2 A AC for 12 min. The count of bacteria damaged by the electric current was reduced from $9.6x10^4$ c.f.u./mL to $1x10^3$ c.f.u./mL (1.98 log RF) after eight hours storage at room temperature. At the end of experiment, the reduction factor of the bacteria was 4.3 log (Table IV).

Experiment 5 was performed to examine only the effect of 1.5 A DC treatment applied together with 2 A AC on the mixed culture of hide bacteria during and after application of the electric treatment but the electric treatments were applied on the mixed culture of bacteria twice. First, 12 min of 1.5 A DC treatment applied together with 2 A AC was applied to the mixed culture of hide bacteria in the liquid medium. Then, this medium was left standing for three hours at room temperature. After the waiting hours, eight min of 1.5 A DC treatment applied together with 2 A AC was applied on the mixed culture of bacteria in the liquid medium, and this medium was left standing for five hours at room temperature. At the end of the first electric treatment, temperature of the test medium reached 26°C and at the end of the second electric treatment reached 30 °C (Table V).

TABLE IV
Values of temperature, voltage, bacterial cell counts and reduction factors of the mixed culture obtained from experiment 4.

Treatment time with electric current (min)	Temperature (°C)	Voltage (V)	Bacterial cell counts (c.f.u./mL)	Reduction factor (Log_{10})
BE	20	-	$2.00x10^7$	7.30
2 DC	21	16.1	$1.28x10^7$	0.19
4 AC	22	23.2	$1.05x10^7$	0.28
6 DC	23	15.3	$5.90x10^6$	0.53
8 AC	24	23.0	$2.10x10^6$	0.98
10 DC	25	15.0	$2.12x10^5$	1.97
12 AC	26	21.7	$9.60x10^4$	2.32
Storage duration (hour)				
1	26	-	$6.30x10^4$	2.50
2	26	-	$5.60x10^4$	2.55
3	25	-	$4.80x10^4$	2.62
4	25	-	$3.20x10^4$	2.80
5	25	-	$2.50x10^4$	2.90
6	25	-	$1.30x10^4$	3.19
7	25	-	$2.00x10^3$	4.00
8	25	-	$1.00x10^3$	4.30

The bacterial cell count was reduced to $9.4x10^4$ c.f.u./mL (2.33 log RF) after the treatment of DC applied together with AC for 12 min. The bacterial cell counts decreased during three hours storage at room temperature but the reduction of the bacterial cell counts (<1 log RF) was fairly low. After the second electric treatment, the bacterial cell count was reduced to $5x10^3$ c.f.u./mL (3.6 log RF). At the end of experiment, the reduction factor of the bacteria was 5.19 log. When the mix culture damaged by the electric current treatment was stored at room temperature for five hours, 1.19 log reduction factor was detected during storage. Data obtained from experiment 5 showed that the bacterial cell counts can be reduced to very low level by two applications of the AC treatment applied together with DC (Table V).

Experiment 6 was accomplished to determine the synergistic effect of DC treatment applied together with AC and bronopol on the mixed culture of hide bacteria. In this experiment, the test medium was left for three hours at room temperature after the electric current treatment. Then, 1 g/L of bronopol was added and the test medium was left for five hours at room temperature. At the end of the first electric treatment, temperature of the test medium reached 26°C and at the end of the experiment was 24 °C (Table VI).

The bacterial cell count was reduced to $9.5x10^4$ c.f.u./mL (2.32 log RF) after the DC treatment applied together with AC for 12 min. The reduction of the bacterial cell counts (<1 log RF) was fairly low during three hours storage at room temperature. Then, 1 g/L of bronopol was added and the bacteria in the

medium were treated with the antibacterial agent for five hours. When the bacterial culture, which was damaged by the electric current in the test medium, was treated with 1 g/L of the test agent, 1.86 log RF (total of 4.43 log RF) of the mixed culture was detected after one hour exposure time. The bacteria damaged by the electric current were completely inactivated with 1 g/L of bronopol after five hours exposure time. The results of this experiment were very similar to the result of experiment 3. The bacteria damaged by the electric current were killed in a shorter time than the time required for their inactivation by only bronopol. These results showed that three hours storage at the room temperature did not change the

TABLE V
Values of temperature, voltage, bacterial cell counts and reduction factors of the mixed culture obtained from experiment 5.

Treatment time with electric current (min)	Temperature (°C)	Voltage (V)	Bacterial cell counts (c.f.u./mL)	Reduction factor (Log_{10})
BE	20		$2.00x10^7$	7.30
2 DC	21	21.0	$1.52x10^7$	0.12
4 AC	22	29.5	$1.15x10^7$	0.24
6 DC	23	15.1	$5.40x10^6$	0.57
8 AC	24	22.1	$2.00x10^6$	1.00
10 DC	25	13.7	$1.83x10^5$	2.04
12 AC	26	20.3	$9.40x10^4$	2.33
Storage duration (hour)				
1	26	-	$7.20x10^4$	2.44
2	26	-	$6.80x10^4$	2.47
3	25	-	$5.20x10^4$	2.59
Treatment time (min)				
2 DC	26	19.3	$3.50x10^4$	2.76
4 AC	27	27.5	$3.00x10^4$	2.82
6 DC	28	16.2	$1.00x10^4$	3.30
8 AC	30	23.3	$5.00x10^3$	3.60
Storage duration (hour)				
1	30	-	$2.00x10^3$	4.00
2	30	-	$1.00x10^3$	4.30
3	29	-	$5.60x10^2$	4.55
4	28	-	$2.50x10^2$	4.90
5	28	-	$1.30x10^2$	5.19

counts of bacterial cell significantly. Therefore, we suggest adding the antibacterial agent into the test medium after the electric current treatment (Table VI).

In addition to DC treatments, AC treatments were used to inactivate microorganisms. Inactivation effect of alternating electric current on *Escherichia coli* ATCC 25922 and fecal *E. coli* MAAG 1405, which was resistant to antibiotics, was examined in water samples collected from Ayamama River, Sarısu River, their discharge points into the sea, and from the Black Sea and Marmara Sea. In addition to water samples, lauryl sulfate broth was used as an organic rich medium. Both test strains were inactivated within 1-3 min in media containing marine waters (1 A), 5-10 min in Ayamama River water (1 A), 5 min in Sarısu River water (0.5 A), 20-35 min in lauryl sulfate broth (1 A) by applying alternating electric current.[29]

In another study, direct electric current was used to inactivate the mixed bacterial culture containing *Escherichia coli*, *Staphylococcus aureus* and *Vibrio parahaemolyticus* at a density of 10^6 c.f.u./mL. Electric current was applied at intervals of 10 ms, 50 ms, 100 ms, 500 ms, 1000 ms, and 2000 ms on the test bacteria, and it was emphasized that Cl_2 gas was

TABLE VI
Values of temperature, voltage, bacterial cell counts and reduction factors of the mixed culture obtained from experiment 6.

Treatment time with electric current (min)	Temperature (°C)	Voltage (V)	Bacterial cell counts (c.f.u./mL)	Reduction factor (Log_{10})
BE	20		$2.00x10^7$	7.30
2 DC	21	17.8	$1.42x10^7$	0.15
4 AC	22	25.1	$1.08x10^7$	0.27
6 DC	23	14.7	$3.55x10^6$	0.75
8 AC	24	20.8	$1.60x10^6$	1.10
10 DC	25	13.7	$1.76x10^5$	2.05
12 AC	26	20.1	$9.50x10^4$	2.32
Storage duration (hour)				
1	26	-	$7.00x10^4$	2.45
2	26	-	$6.70x10^4$	2.47
3	25	-	$5.40x10^4$	2.57
Treatment time with 1 g/L of Bronopol (hour)				
1	25		$7.50x10^2$	4.43
2	25	-	$2.30x10^2$	4.94
3	25	-	$5.00x10^1$	5.60
4	24	-	$3.00x10^1$	5.82
5	24	-	-	7.30

not produced during the application except 2000 ms. It was stated that although *V. parahaemolyticus* was completely destroyed by 263 mA electric treatment within 100 ms in the test medium containing 0.9% NaCl, the other two bacteria stayed alive. Researchers mentioned that all bacteria were killed at 526 mA. Researchers observed that 263 mA DC applied for 100 ms caused pores in the membranes of all of the test bacteria and that the natural morphology of *V.parahaemolyticus* has changed.[30]

The researchers showed that the electric current applied in combination with biocides was more effective than when it was used independently against biofilm bacteria. Investigators observed that the 24 h biofilms of *P. aeruginosa* were not inactivated via the electric current alone. The application of electric current (9 mA/cm^2 current density for 12 h.) together with antibiotics (ciprofloxacin, polymyxin B and tobramycin) inhibited the bacteria in the biofilms. When the electrical current was applied, the action of the antibiotics was enhanced. Therefore, researchers emphasized that low electrical currents increased the activity of antimicrobials against biofilm bacteria.[31]

It has been stated that bronopol containing bactericides is very effective against different microorganisms and it may be used in different applications. 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. It is used at 0.01 to 0.1% in hand and face creams, shampoos, hair dressings, mascaras, bath oils, pharmaceutical products and household products (fabric conditioners and washing detergents). Bronopol forms disulfide bonds with thiol groups to denature proteins, and it inhibits dehydrogenase activity.[24]

It is believed that electric current may electrolyze molecules on the cell surface. Electric currents also affect the orientation of membrane lipids and consequently cell viability. It has been proposed that bacterial inactivation may be due to oxidation of vital cellular constituents during exposure to electric current. In addition, it has been explained that hydrogen peroxide, ozone, free chlorine and chlorine dioxide are produced by electrodes during electric current treatment in the presence of chloride ions in the medium. These toxic oxidants have been suggested as the main bactericidal agents. Researchers observed that DC on microorganisms in seawater caused considerable structural damage at cellular level and irreversible cell membrane rupture in different parts of cell membrane with the apparent leakage of intracellular contents. When electrochemically generated oxidants are present, these pores may allow the oxidants free access to the interior of the cell, aiding the inactivation process. It has been proposed that electric current causes bacterial cell membrane damage such as growth of the membrane surface shape fluctuations and molecular rearrangements, thus leading to a membrane discontinuity and pore expansion which can result in an irreversible membrane breakdown.[32-35]

CONCLUSION

This is the first study to investigate the synergistic effect of a combined electric current treatment using both DC and AC, followed by bronopol treatment on the mixed culture of Gram positive bacteria (*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 liquid medium containing organic substance and 2% NaCl. This treatment system proved to be a very effective way to inactivate Gram positive bacteria, Gram positive endospore-forming bacteria and Gram negative bacteria in the liquid medium containing organic substance and 2% NaCl such as soak liquors. While the bacterial cell count of the mixed culture was reduced to the reasonable level at which the bacteria cannot damage the hide after 12 min treatment with 2.0 A AC applied together with 1.5 A DC, five hours exposure time with bronopol was necessary to reduce the bacterial cell count of the mixed culture to the reasonable level. Based on the results of our experiment, we concluded that bronopol should be added into the hide soak liquor after treatment of the bacteria with the electric current. Our results showed that the electric current treatment helped penetration of bronopol into the damaged bacterial cells and increased the efficacy of bronopol. When the mixed culture of hide bacteria was damaged with AC applied together with DC, low concentration of antibacterial agent was enough to kill the bacteria in the liquid medium containing organic substance and 2% NaCl such as hide soak liquor. The pH of the test media did not change during the treatments. In all electric treatments, the experiment was carried out at the constant DC and AC values. As the voltage was decreased, consumed power was continuously decreased. This made us aware that when the mixed culture of bacteria was killed the energy required to do so also decreased proportionally. As a conclusion, a combined electric current treatment using both DC and AC, followed by bronopol treatment can be used as a simple and effective method for inactivating proteolytic and lipolytic different bacterial populations in hide-soak liquors.

ACKNOWLEDGEMENTS

We wish to express our gratitude to Dr. Emel Aslan Con for her excellent research on isolation and identification of bacteria from salted hides. We thank Dr. Martin Louis Duncan for valuable proofreading of our manuscript. This manuscript was dedicated to our wonderful teachers Professor Meral Unal, Professor Ali Cirpici and Professor Engin Ozhatay.

REFERENCES

1. Woods, D.R., Atkinson, P.; The Bacteriology of the Leather Industry. *JALCA* **5,** 360-383, 1970.
2. Anderson, R.; Wet Processing, Beamhouse Processing. *Leather*. 18-24, 1992.
3. Rangarajan, R., Didato, T.D., Bryant, S.; Measurement of Bacterial Populations in Typical Tannery Soak Solutions by Traditional and New Approaches. *JALCA* **98**, 477-485, 2003.
4. Stockman, G., Didato, D.T., Steele, S., Black, C., Allison, R.; Soaking: Balancing Operational and Quality Issues Using Both Fresh and Brine Cured Hides. *JALCA* **103**, 76-85, 2008.
5. Birbir, Y., Ugur, G., Birbir, M.; Inactivation of Bacterial Population in Hide Soak Liquors via Direct Electric Current. *J. Electrostat*. **66**, 355-360, 2008.
6. Berber, D., Birbir, M.; Examination of Bacterial Populations in Salt, Salted Hides, Soaked Hides and Soak Liquors. *JALCA* **105**, 320-326, 2010.
7. Aslan, E., Birbir, M.; Examination of Efficiency and Sufficiency of Salt-pack Curing Method. *J. Soc. Leather Technol. Chem*. **95**, 98-103, 2011.
8. Madigan, M.T., Martinko, J.M., Dunlop, P.V., Clark, D.P.; Brock Biology of Microorganisms. 13th ed., Pearson Education, Benjamin Cummings, San Francisco, USA, 2012.
9. Balows, A.; Manual of Clinical Microbiology, 5th Ed., American Society for Microbiology, Washington, D.C., 1991.
10. Woods, D. R., Atkinson, P., Cooper, D.R., Galloway, A.C.; The Microbiology of Curing and Tanning Processes.Part II. Analysis of Aerobic Bacteria in Static Hide Brining. *JALCA* 65, 164, 1970.
11. Woods, D.R., Atkinson, P., Cooper, D.R.; The Microbiology of Curing and Tanning Processes. Part III: Analysis of Aerobic Bacteria in Agitated Hide Brining. *JALCA* **65,** 410-418, 1970.
12. Veyselova, C., Birbir, M., Berber, D.; Quaternary Ammonium Compound as Bactericide in Hide Brine Curing and Presoaking Processes. *JALCA* **107**, 10, 336-341, 2012.
13. Berber, D., Birbir, M., Hacioglu, H.; Efficacy Assessment of Bactericide Containing Didecyldimethyl-ammonium Chloride on Bacteria Found in Soak Liquor at Different Exposure Times. *JALCA* **105**, 354-387, 2010.
14. Aslan, E. and Birbir, M.; Examination of Gram Negative Bacteria on Salt-Pack Cured Hides. *JALCA* **107**, 106-115, 2012.
15. Aslan, E. and Birbir, M.; Examination of Gram Positive Bacteria on Salt-Pack Cured Hides. *JALCA* **106**, 372-380, 2011.
16. Bailey, D.G.; Gamma Radiation Preservation of Cattle Hides, A New Twist on An Old Story. *JALCA* **70**, 248-260, 1975.
17. Bailey D.G., DiMaio, G. L., Gehring, A. G., Ross, G. D.; Electron Beam Irradiation Preservation of Cattle Hides in a Commercial-scale Demonstration, *JALCA* **96,** 382-392, 2001.
18. Birbir, Y., Birbir, M.; Inactivation of Extremely Halophilic Hide-damaging Bacteria via Low-level Direct Electric Current. *J. Electrostat*. **64,** 791-795, 2006.
19. Birbir, Y., Degirmenci, D. and Birbir, M.; Direct Electric Current Utilization in Destruction of Extremely Halophilic Bacteria in Salt That is Used in Brine Curing of Hides. *J. Electrostat*. 92 (2), **53-58,** 2008.
20. Muthusubramanian, L., Mitra, R.B.; A Cleaner Production Method for the Synthesis of Bronopol A Bactericide That is Useful in Leather Making. *J. Clean. Prod*. **14**, 536-538, 2006.
21. Ur-banski, T., Eckstein, Z., Wojnocoska, H.; Bull Acad Polon. *Sci. Classe III*. 4, 461-463, 1956.
22. Ur-banski, T., Eckstein, Z., Wojnocoska, H.; *Roczniki Chem*. 31, 93-100, 1957.
23. Block, S.S.; Disinfection, Sterilization and Preservation, 5th Ed., Lippincott Williams and Wilkins, Philadelphia, USA, 403, 2001.
24. Geis, P. A.; Cosmetic Microbiology: A Practical Approach, Second Edition, Taylor and Francis Group, New York, 235-236, 2006.
25. Costerton, J.W., Ellis, B., Lam, K., Johnson, F., Khoury, A.E.; Mechanism of Electrical Enhancement of Efficacy of Antibiotics in Killing Biofilm Bacteria. *Antimicrob. Agents Chemother*. **38**, 2803-2809, 1994.
26. Khoury, A. E., Lam, K., Ellis, B., Costerton, J. W.; Prevention and Control of Bacterial Infections Associated with Medical Devices. *Am. Soc. Artif. Intern. Org. J*. 38, 174-178, 1992.
27. Birbir, M., Dolek, N.; Examination of Effect Following Application of Direct and Alternating Electric Current on Gram Positive and Gram Negative Bacteria. FEN C-YLP-161111-0298, BAPKO, 2013.
28. Park, J.C., Lee, M.S., Lee, D.H., Park, B.J., Han, D.W., Uzawa, M., Takatori, K.; Inactivation of Bacteria in Seawater by Low-amperage Electric Current. *Appl. Environ. Microbiol*. **69**, 2405-2408, 2003.
29. Birbir, M., Hacıoğlu, H., Birbir, Y., Altug, G.; Inactivation of *Escherichia coli* by Alternating Electric Current in Rivers Discharged into Sea. *J. Electrostat*. **67**, 4, 640-645, 2009.
30. Jin, S. C., Yoon, H.S., Woo, Y. I., Lee, M. H., Vagaska, B., Kim, J. S., Uzawa, M. Park, J.C.; Selective Sterilization of *Vibrio parahaemolyticus* from a Bacterial Mixture by Low-amperage Electric Current. *J. Microbiol. Biotechnol*. **19**, 537-541, 2009.

31. Jass, J., Lappin-Scott, H. M., The Efficacy of Antibiotics Enhanced by Electrical Currents Against *Pseudomonas aeruginosa* Biofilms. *Journal of Antimicrobial Chemotherapy*. **38**, 987-1000, 1996.
32. Dimitrov, D.S., Jain, R. K.; Membrane Stability. *Biochim. Biophys. Acta*. **779**, 437-468, 1984.
33. Drees, K.P., Abbaszadegan, M., Maier, R. M.; Comparative Electrochemical Inactivation of Bacteria and Bacteriophage. *Water Res*. **37**, 2291-2300, 2003.
34. Davis, C.P., Shirtliff, M.E., Trieff, N.M., Hoskins, S.L., Warren, M.M.; Quantification, Qualification, and Microbial Killing Efficiencies of Antimicrobial Chlorine-based Substances Produced by Iontophoresis. *Antimicrob. Agents Chemother.* **38**, 2768-2774, 1994.
35. Liu, W.K., Brown, M.R.W., Elliot, T.S.J.; Mechanisms of the Bactericidal Activity of Low Amperage Electric Current (DC). *J. Antimicrob. Chemother.* **39**, 687-695, 1997.