# Direct Electric Current Method for Eradication of Slightly Halophilic *Bacillus* Species Isolated from Salted Hides

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**Summary**

Raw animal skin/hide samples are salted and stored for long periods of time when they cannot be used immediately after skinning process. Salting prevents the growth of bacteria on hides by making the environment unfavorable for enzymatic activities. This method is suitable for non-salt tolerant micro-organisms. However, halophilic bacteria, which are require salt, grow under these conditions, resulting in damage to the skin structure and reducing skin quality. Considering this situation, in our study, each slightly halophilic bacterial strain, isolated from salted sheepskins, and their mixed cultures prepared from these isolates (TKD1a-*Bacillus haynesii*, TKD1b-*Bacillus rugosus*, TKD2a-*Bacillus safensis*, TKD7b-*Bacillus aerius*, TKD9a-*Bacillus kochii*) were treated with 2.3 A direct electric current (DC) in the presence of 5% NaCl. It was determined that TKD9a-*Bacillus kochii* was killed completely in 21 minutes, TKD1a-*Bacillus haynesii*, TKD1b-*Bacillus rugosus*, TKD2a-*Bacillus safensis*, TKD7b-*Bacillus aerius* and the mixed culture of these five bacteria were killed completely in 24 minutes. While the temperature of all test media was 24°C before the experiment, it increased in TKD1b-*Bacillus rugosus* and TKD9a-*Bacillus kochii* test media after 1 minute of the DC application. The pH value of the medium increased from 6.7 to 7.7 due to application of the DC. The voltage levels of the test medium varied according to the duration of the DC application and the bacterial species. As a result, it was determined that DC application can be a method for controlling slightly halophilic bacterial strains used in our study.

## 1 INTRODUCTION

Animal skins/hides are the first line of defense that protects the body from environments. Skins/hides also play a role in balancing body temperature. When removed from animals after slaughter, this important structure becomes raw material for the leather industry.[1,2] Raw hides contain 29% structural protein (primarily collagen) and 1.7% non-structural protein (albumin, globulin, mucoid and mucin), 2% fat, 0.5% mineral salt and 64% water.[3,4] The high water content in the hide structure creates a perfect environment for micro-organisms to reproduce in a period of time as short as 5-6 hours.[5] In order to reduce this high water content, hides and skins are stored with different preservation methods. The most common method is salt curing of raw hides/skins.[5-7] In this curing method, salt conditioning creates a bacteriostatic effect, causing plasmolysis and preventing the development of bacteria.[8] Although the salt curing method applied to skins and hides may prevent the growth of non-halophilic bacteria, this method supports the growth of halophilic micro-organisms and halotolerant bacteria.[5,9,10] A previous investigation used molecular techniques to demonstrate that halophilic bacteria and archaea cause red spots and various pigment discolorations on the salted-skin samples.[11] In that study, moderately halophilic bacteria and extremely halophilic archaea were isolated from deteriorated salted-skin samples obtained from a warehouse in Spain and analysed using 16S rRNA gene sequencing. The number of moderately halophilic bacteria was determined to be $10^5$-$10^8$CFU/g, and the number of extremely halophilic archaea was determined to be $10^5$-$10^7$CFU/g. In addition, morphological changes such as hair loss and bad odour formation were detected in skin samples, and it was revealed that halophilic bacteria and archaea caused microbial damage to the skin because of their proteolytic and lipolytic activities.[11] In a separate study examining sheepskin samples preserved with salt, electron microscopy showed that the proteolytic and lipolytic halophilic archaea isolated from skin samples caused severe damage to the skin over a storage period of 47 days.[12] Furthermore, it has been demonstrated in previous study that halophilic micro-

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organisms cause collagen damage, unpleasant odour, and hair looseness. These defects thus decrease the hides' economic value.[13]

Not all halophilic micro-organisms can survive the same salt concentration. These micro-organisms are classified into different categories based on the salt concentrations at which they can grow most effectively. Slightly halophilic bacteria used in our experiment are micro-organisms that grow at an optimum salt concentration of 0.2-0.5M (1-3%).[14] Slightly halophilic bacteria have been previously isolated from offshore oil wells,[15] surface sediment in Hong Kong,[16] seawater samples in Korea,[17] saline-alkaline soil samples in China[18] and from diverse sources such as salted sheepskins.[19] In an earlier study, the slightly halophilic bacterial strain *Cobetia crustatorum* was isolated from Jeotgal, a traditional Korean salted fermented product. *C. crustatorum* was found to be Gram-negative and showed optimum growth at 6.5% NaCl concentration and pH5-6. Additionally, it can utilise many sugars and amino acids.[20] Researchers have likewise isolated Gram-positive, obligate aerobic *Bacillus salacetis sp. nov.,* slightly halophilic bacterial strain from traditional salted shrimp paste, which is widely consumed in Thailand. It has been reported that it can grow at optimum values of pH7.5, 37℃ and 1-3% NaCl concentration.[21] In the aforementioned reports, it is understood that slightly halophilic bacterial isolates survive at relatively low salt concentrations.

Although various antimicrobial agents have been previously applied to prevent the growth of halophilic micro-organisms on salted hides and skins, the problem of antimicrobial resistance has emerged due to their inappropriate and random use.[5,9,11,22-24] Hence, different methods were suggested for the preservation of hides and skins. These methods include application of chemicals such as sodium sulphate[6] and potassium chloride;[25] the use of lichen extracts[24] and plant extracts;[23,26] short-term preservation of skins under controlled conditions by applying vacuum;[27] and the use of bacteriocin obtained from *Lactobacillus plantarum*.[28]

Several studies have examined the inactivation of micro-organisms using DC in the food industry, medical applications, and the leather industry. The bactericidal effects of DC and alternating current (AC) have been demonstrated in several environments, including milk,[29] salt solutions,[30] human skin,[31] synthetic urine,[32] water[33] and intravenous catheters.[34] In the leather industry, researchers emphasised that inactivation of extremely halophilic archaea and moderately halophilic bacteria with DC and AC is a very effective method.[35-39] It was reported that while the mixed cultures of the extremely halophilic archaea found in a liquid medium containing organic matter and 25% NaCl ($10^7$CFU/mL) were killed using 0.5 A DC within 10 minutes, only protease-producing extremely halophilic archaea were inactivated *via* 0.5A DC within 5 minutes. However, only lipase or both protease and lipase-producing extremely halophilic archaea were killed *via* 0.5 A DC in 20 minutes.[35] In another study, it was discovered that 0.5A DC inactivated extremely halophilic archaea in curing salt samples dissolved in the brine solution ($10^4$-$10^5$CFU/g) within 1 minute.[36]

In addition, the mixed culture of moderately halophilic bacteria mainly produces protease and lipase (*Chromohalobacter israelensis, C. canadensis, Halomonas halodenitrificans, H. halmophila* and *Staphylococcus nepalensis*) in the brine containing 10% NaCl was killed by 0.5A DC in 10 minutes, 1.0A DC in 5 minutes, 1.5A DC in 3 minutes, and 2.0A DC in 1 minute, the mixed culture of moderately halophilic bacteria was killed by 0.5A AC in 20 minutes, 1.0A AC in 15 minutes, 1.5A AC in 10 minutes, and 2.0A AC in 5 minutes.[39] In a later experiment, proteolytic and lipolytic haloversatile bacteria in 15% NaCl solution ($3.6\times10^6$CFU/mL) were completely inactivated *via* 2.5A DC within 21 minutes.[40] As mentioned in the literature, while the inactivation of moderately halophilic bacteria, extremely halophilic archaea and haloversatile bacteria *via* the DC treatments has been accomplished, the inactivation of slightly halophilic bacterial strains isolated from salted sheepskins *via* DC treatments has not yet been examined. Therefore, the goal of this study is to investigate the inactivation effect of 2.3A DC on slightly halophilic bacterial strains isolated from salted sheepskins that exhibit enzymatic activities.

## 2 EXPERIMENTAL PROCEDURES

### 2.1 Test bacteria

The test isolates used in the present study were slightly halophilic species such as *Bacillus haynesii* isolate TKD1a*, Bacillus rugosus* isolate TKD1b*, Bacillus safensis* isolate TKD2a*, Bacillus aerius* isolate TKD7b*, Bacillus kochii* isolate TKD9a. Those isolates were previously isolated from the salted-sheepskin samples, characterised by both conventional and molecular methods in a previous study.[19] The isolates stored in glycerol (20%, v/v) at -24°C were streaked on the Complex Agar Medium containing 3% total salt (w/v, SW3) and 0.5% (w/v) yeast extract.[41] The inoculated petri plates were incubated at 37°C for 24 hours. Gram staining method using 3% NaCl solution was performed to check purity of test isolates.[42]

### 2.2 Application of direct electric current on the test isolates

Five slightly halophilic salted-sheepskin isolates (*Bacillus haynesii* isolate TKD1a, *Bacillus rugosus* isolate TKD1b, *Bacillus safensis* isolate TKD2a, *Bacillus aerius* isolate TKD7b, *Bacillus kochii* isolate TKD9a) were used in electric current applications. Each isolate was grown in liquid Complex Medium (containing 3% total salt) for 24 hours at 37°C. After incubation, the population of the isolates was adjusted to $10^6$CFU in one mL. Next, their mixed culture was prepared by adding 4mL of each test isolate. 20mL of each test micro-organism and 20mL of the mixed culture were separately added into the electrolysis cells

(a glass beaker, two internally attached platinum wire electrodes) containing 5% NaCl + organic matter (180mL) ($10^5$CFU/mL) (Fig. 1).[12,43] At the beginning of the experiment, 100µL test media were taken from each electrolysis cell, then diluted with sterilised physiological saline water (3% NaCl) and spread over the Complex Agar Medium. The plates were incubated at 37°C for 24 hours. The visible colonies grown on Complex Agar plates were counted after incubation. Each electrolysis cell was separately exposed to 2.3A direct current for 24 minutes. During the experiment, 100µL of liquid medium was taken from the glass beaker at intervals of 1, 3, 6, 9, 12, 15, 18, 21, and 24 minutes. Direct and diluted $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$ suspensions were spread over Complex Agar plates (containing 3% total salt). After incubation for 24 hours at 37°C, the visible colonies were counted. Before starting the experiment, the temperature and pH were measured as 24°C and 6.7, respectively.

## 3 RESULTS AND DISCUSSION

We previously determined that all of the bacterial strains used in our study have protease activity.[19] Protease enzyme activity causes deterioration in animal skins and digests the protein content found in the structure of the skin.[44] Anderson (1949) detected proteolytic *Bacillus* species in salted-skin samples collected from Australia and New Zealand. In another study, *Bacillus subtilis, B. pumilus, B. cereus, B. sphaericus, B. firmus, B. megaterium, B. licheniformis, B. brevis* and *B. laterosporus* were detected in 225 leather samples taken from freshly skinned hides, one-week salted hides, two-month salted hides, lime-treated hides, soaked hides, chrome-tanned hides, and processed finished hides. That research detected the presence of *Bacillus* species in all skin samples.[45] In a study examining 10 salted leather samples from the United Kingdom, Australia and Türkiye, 111 *Bacillus* isolates such as *B. laterosporus, B. amyloliquefaciens, B. pumilus, B. firmus, B. lentus, B. cereus, B. licheniformis, B. megaterium, B. mycoides, B. subtilis* and *B. thuringiensis* were identified from leather samples.[46] Polkade (2007)[47] isolated bacterial strains of the genus *Bacillus* from fresh buffalo hides collected immediately after slaughter and found that these bacteria exhibited higher hydrolytic activity compared to other bacterial strains. The study reported that the predominant bacterial genera in the leather samples were *Bacillus, Stenotrophomonas, Acinetobacter* and *Escherichia*. It was specifically found that *Bacillus megaterium* strain had proteolytic activity strong enough to damage the hides.[47]

*Bacillus* species produce endospores that are highly resistant to many adverse conditions. The endospore structure is highly resistant to thawing, heat, oxidising agents, ultraviolet radiation, gamma rays, physical abrasion, over-drying, toxic chemicals and digestive enzymes.[48] Different alternatives are needed to prevent this situation and also prevent these bacterial strains from contaminating hides. Therefore, in our study, TKD1a-*Bacillus haynesii,* TKD1b-*Bacillus rugosus,* TKD2a-*Bacillus safensis,* TKD7b-*Bacillus aerius* and TKD9a-*Bacillus kochii* bacterial isolates were initially both treated separately and, subsequently, their mixed cultures were treated with 2.3A DC in the presence of 5% NaCl. After 15 minutes of the DC application, the total counts of *Bacillus haynesii* isolate TKD1a decreased from $5.1\times10^5$ to $1\times10^2$CFU/mL. At 24 minutes, *B. haynesii* isolate TKD1a was found to be completely killed (Table I). After 15 minutes of the DC application, the total counts of *B. rugosus* isolate TKD1b bacterial strain decreased from $2.2\times10^5$ to $5\times10^1$CFU/mL. *B. rugosus* isolate TKD1b were detected to be totally dead within 24 minutes (Table I). After 15 minutes of treatment with the DC application, the total count of *B. safensis* isolate TKD2a decreased from $5\times10^5$ to $9\times10^1$CFU/mL. After 24 minutes, all *B. safensis* isolate TKD2a cells were killed (Table I). After 15 minutes of treatment with the DC application, the total count of *B. aerius* isolate TKD7b decreased from $6.7\times10^5$ to $8\times10^1$CFU/mL. After 24 minutes, all *B. aerius* isolate TKD7b cells were killed (Table I). After 15 minutes of treatment with the DC application, the total count of *B. kochii* isolate TKD9a decreased from

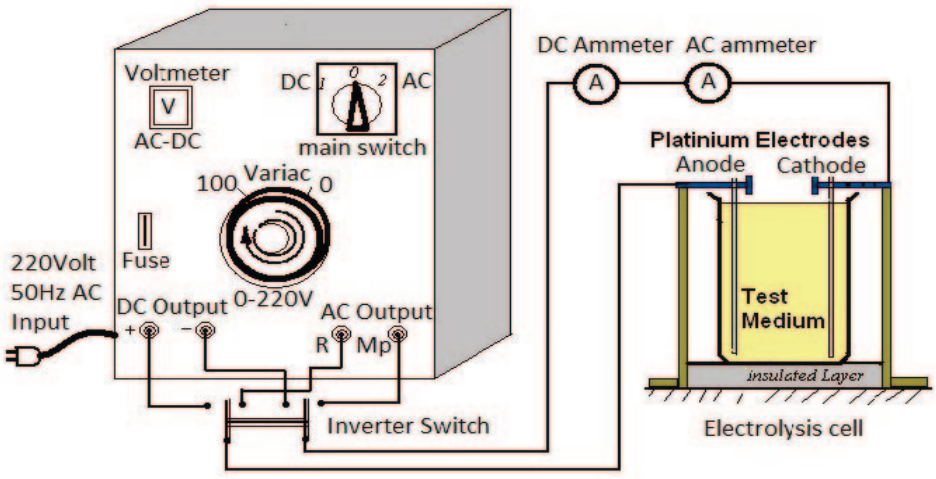



**Figure 1**. Direct current electrolysis cell system. R: phase, Mp: ground.[12,43]

**TABLE I**
**Bacterial Counts of Each Slightly Halophilic Isolate and Their Mixed Culture Treated with 2.3 A Direct Electric Current in 5% NaCl Saline Solution and Organic Content**

| - | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Species names of test bacteria | *Bacillus haynesii* | *Bacillus rugosus* | *Bacillus safensis* | *Bacillus aerius* | *Bacillus kochii* | The mixed culture |
| Isolate code | TKD1a | TKD1b | TKD2a | TKD7b | TKD9a | |
| BE* | $5.1\times10^5$ | $2.2\times10^5$ | $5\times10^5$ | $6.7\times10^5$ | $2.3\times10^5$ | $7.3\times10^5$ |
| DCT**(min) | | | | | | |
| 1 | $2.4\times10^5$ | $1.1\times10^5$ | $3.1\times10^5$ | $3\times10^5$ | $1.3\times10^5$ | $4.1\times10^5$ |
| 3 | $6.3\times10^4$ | $5.4\times10^4$ | $8.3\times10^4$ | $1.1\times10^5$ | $1.1\times10^5$ | $2.2\times10^5$ |
| 6 | $1.4\times10^4$ | $1.9\times10^4$ | $2.1\times10^4$ | $5\times10^4$ | $4\times10^4$ | $1\times10^5$ |
| 9 | $7.5\times10^3$ | $6.1\times10^3$ | $2.5\times10^3$ | $3\times10^3$ | $2.6\times10^3$ | $5.4\times10^4$ |
| 12 | $1.5\times10^3$ | $1.3\times10^2$ | $6.2\times10^2$ | $5.2\times10^2$ | $2.2\times10^2$ | $2.6\times10^3$ |
| 15 | $1\times10^2$ | $5\times10^1$ | $9\times10^1$ | $8\times10^1$ | $7\times10^1$ | $8.5\times10^2$ |
| 18 | $8\times10^1$ | $3\times10^1$ | $7\times10^1$ | $6\times10^1$ | $1\times10^1$ | $4.4\times10^2$ |
| 21 | $1\times10^1$ | $2\times10^1$ | $2\times10^1$ | $3\times10^1$ | - | $1.3\times10^1$ |
| 24 | - | - | - | - | - | - |

*BE: Before Experiment; **DCT:Direct Electric Current Treatment

$2.3\times10^5$ to $7\times10^1$CFU/mL. *All B. kochii* isolate TKD9a cells were dead after 21 minutes (Table I). Following 15 minutes of treatment with the DC application, the total count of the mixed culture decreased from $7.3\times10^5$ to $8.5\times10^2$CFU/mL. At 24 minutes, with 2.3 A electric current, all of the mixed culture was certainly killed (Table I).

The temperature of all test media was 24°C before the experiment and remained the same in *B. haynesii* isolate TDK1a, *B. safensis* isolate TKD2a, *B. aerius* isolate TKD7b and mixed culture media after 1 minute of electrical treatment. At the end of the experiment (at 24 minutes), while the temperature of the test media of *B. safensis* isolate TKD2a and the mixed culture reached 36°C, the temperature values of the test media of *B. rugosus* isolate TKD1b, *B. aerius* isolate TKD7b and *B. kochii* isolate TKD9a reached 35°C. The temperature value of the test medium of *B. haynesii* isolate TDK1a was measured as 33°C after 24 minutes (Table II). The pH values of all test media increased from 6.7 to 7.7 at the end of the experiment. Voltage levels of the test media changed according to the duration of the electric current treatment and the bacterial strain. However, all voltage levels decreased at the end of the experiments (Table II). The observed decrease in voltage and increase pH during the experiment may be a result of bacterial damage and chemical changes induced by the DC treatment and electrolysis processes in the solution. Additionally, the rise in temperature of the test media was also caused by the duration of DC treatment.

Electric current has been used to kill various bacteria.[49] While early studies focused more on bactericidal effects with the application of high electrical voltages, the studies in the past few years have revealed that low electrical voltages and currents can effectively kill biofilms.[49] For example, the effects of low electric currents ranging from 10 to 200mA on the viability of *Escherichia coli* bacteria were investigated. As a result of that study, it was found that currents as low as 25mA can kill the bacteria.[50] The effects of electric fields on micro-organisms are often

**TABLE II**
**Voltage, pH and Temperature Values of the Test Medium during the Electric Current Treatments**

| | 1 | | | 2 | | | 3 | | | 4 | | | 5 | | | 6 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Species names of test bacteria | *Bacillus haynesii* | | | *Bacillus rugosus* | | | *Bacillus safensis* | | | *Bacillus aerius* | | | *Bacillus kochii* | | | The mixed culture | | |
| Isolate code | TKD1a | | | TKD1b | | | TKD2a | | | TKD7b | | | TKD9a | | | | | |
| | **V** | **pH** | **°C** | **V** | **pH** | **°C** | **V** | **pH** | **°C** | **V** | **pH** | **°C** | **V** | **pH** | **°C** | **V** | **pH** | **°C** |
| **BE** | - | 6.7 | 24 | - | 6.7 | 24 | - | 6.7 | 24 | - | 6.7 | 24 | - | 6.7 | 24 | - | 6.7 | 24 |
| **DCT (min)** | | | | | | | | | | | | | | | | | | |
| **1** | 15.9 | 6.7 | 24 | 20.9 | 6.7 | 25 | 18.0 | 6.7 | 24 | 18.0 | 6.7 | 24 | 18.0 | 6.7 | 26 | 16.5 | 6.7 | 24 |
| **3** | 15.8 | 6.8 | 25 | 19.4 | 6.8 | 26 | 17.5 | 6.8 | 25 | 17.8 | 6.8 | 25 | 17.5 | 6.8 | 28 | 15.1 | 6.8 | 26 |
| **6** | 13.7 | 6.9 | 26 | 17.4 | 6.9 | 28 | 17.0 | 6.9 | 27 | 16.5 | 6.9 | 26 | 17.4 | 6.9 | 29 | 14.0 | 6.9 | 27 |
| **9** | 13.5 | 7.0 | 27 | 16.6 | 7.0 | 29 | 16.9 | 7.1 | 28 | 16.0 | 7.0 | 28 | 17.3 | 7.0 | 30 | 13.9 | 7.1 | 29 |
| **12** | 13.3 | 7.1 | 28 | 15.9 | 7.1 | 30 | 16.7 | 7.2 | 29 | 15.5 | 7.2 | 30 | 17.2 | 7.1 | 31 | 13.8 | 7.2 | 31 |
| **15** | 13.1 | 7.3 | 29 | 15.8 | 7.2 | 31 | 16.0 | 7.3 | 31 | 15.3 | 7.3 | 31 | 16.7 | 7.3 | 32 | 13.6 | 7.4 | 33 |
| **18** | 12.9 | 7.4 | 30 | 15.7 | 7.4 | 32 | 16.0 | 7.5 | 33 | 15.2 | 7.4 | 33 | 16.6 | 7.4 | 33 | 13.6 | 7.5 | 34 |
| **21** | 12.8 | 7.5 | 31 | 15.7 | 7.6 | 33 | 16.0 | 7.6 | 35 | 15.2 | 7.6 | 34 | 16.5 | 7.5 | 34 | 13.6 | 7.6 | 35 |
| **24** | 12.8 | 7.7 | 33 | 15.7 | 7.7 | 35 | 16.0 | 7.7 | 36 | 15.1 | 7.7 | 35 | 16.4 | 7.7 | 35 | 13.5 | 7.7 | 36 |

characterised by changes in the physiology of cells or by disruptions in the inflow and outflow of substances by affecting cell membrane permeability.[51] Generally, microbial damage increases in proportion to the intensity of the electric current and duration of exposure. Irreversible damage may occur in micro-organisms exposed to intense electric current.[52]

There are reports of electric current being used to inhibit bacteria and archaea isolated from different media. In a previous study, researchers separately examined the inactivation of cells of *Listeria monocytogenes* in the brine (14% NaCl) using three different electric currents (1A, 2.5A and 5A). The experiment results showed that higher current levels had a greater killing effect on *L. monocytogenes* population in the brine. It was also explained that higher current level caused higher free chloride concentrations in the storage tank and the treatment chamber.[53] Researchers emphassed that this could be the main reason for the faster killing rate in the treatment chamber.

In a study in which mixed cultures of moderately halophilic bacterial strains (*B. pumilus, Staphylococcus saprophyticus, B. licheniformis, Idiomarina loihiensis* and *Gracilibacillus dipsosauri)* were placed in a liquid medium containing 25% NaCl and then exposed to direct and alternating electric current (0. 5, 1.0, 1.5 and 2.0A), it was found that the mixed culture was killed within 1 minute at 0.5A direct current and 1.5A alternating current.[38] Another experiment conducted a direct electric current of 2.2A through 15% NaCl solution containing a mixed culture of haloversatile bacterial species (*Alkalihalobacillus clausii, Bacillus amyloliquefaciens, B. cereus, B. licheniformis, B. paralicheniformis, B. pumilus, B. sonorensis, Bhargavaea ginsengi, Kocuria rosea, Micrococcus luteus, Oceanobacillus polygoni, Ornithinibacillus scapharcae, Solibacillus silvestris, Staphylococcus hominis subsp. hominis, S. nepalensis, S. warneri, Virgibacillus proomii*) for 25 minutes. The result was that the number of haloversatile bacteria decreased within 16 minutes, and the bacteria died completely within 19 minutes.[54] These experimental results showed that when the salt concentration in the electrolysis cells containing bacteria treated with DC was high, the inactivation of bacteria occurred at a shorter exposure time.

## 4 CONCLUSIONS

This study is the first to apply direct electric current to slightly halophilic bacterial isolates such as *B. haynesii isolate* TKD1a*, B. rugosus isolate* TKD1b*, B. safensis isolate* TKD2a*, B. aerius isolate* TKD7b*, B. kochii* isolate TKD9a and their mixed cultures. By conducting 2.3A direct electric current through a 5% NaCl solution, *B. kochii* isolate TKD9a bacterial strain was completely eliminated in 21 minutes. *B. haynesii* isolate TKD1a, *B. rugosus* isolate TKD1b, *B. safensis* isolate TKD2a, *B. aerius* isolate TKD7b bacterial strains, and the mixed culture of these five bacteria were completely eliminated in 24 minutes. *Bacillus* species with high enzymatic activity and environmental resilience cause cross-contamination *via* salt samples, tanning equipment, or tannery technicians, thereby contaminating and degrading hides. In conclusion, we propose that the application of 2.3A direct electric current could serve an alternative method for controlling slightly halophilic *Bacillus* species in the leather industry.

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