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Application of Hydrogen Peroxide on the Bacterial Control of Seaweed, *Capsosiphon fulvescens* (Mesaengi)

Du-Woon Kim, Mi-Jung Kim, Tai-Sun Shin, Sun-Jae Kim and Bok-Mi Jung†

Division of Food Science and Aqualife Medicine
Chonnam National University, Yeosu 550-749, Korea

해조류 매생이(*Capsosiphon fulvescens*)의 저장기간 연장을 위한 과산화수소의 활용

김두운 · 김미정 · 신태선 · 김선재 · 정복미†

전남대학교 식품수산생명의학부

Abstract

***Bacillus subtilis* subsp. *subtilis* constitutes 90% of the total viable bacteria present on *Capsosiphon fulvescens*. We found that hydrogen peroxide (50 ppm) and NaOCl (50 ppm) were more effective than electrolyzed water (EW, 50 ppm) against *B. subtilis* subsp. *subtilis* that was isolated from this seaweed. Relative to a control, 50 ppm hydrogen peroxide reduced the total viable population by 1.8±0.4 log CFU/g, whereas 50 ppm EW increased the total viable population by 1.7±0.5 log CFU/g. CFUs were evaluated following 30 days of storage at 4°C using air- and vacuum-packaging. Samples treated with 50 ppm hydrogen peroxide and NaOCl showed a 1.6±0.1-fold decrease in initial hardness (7.9 x10^6 dyne/cm^2), while the samples treated with 50 ppm EW had a 2.1±0.1-fold decrease in initial hardness (7.9 x10^6 dyne/cm^2). Again, measurements were performed after storage at 4°C for 20 days. This study indicates that *B. subtilis* subsp. *subtilis* is the most common contaminant in aerobically or anaerobically packaged seaweed and should therefore be the main target for quality control during long-term storage. Hydrogen peroxide and NaOCl are more effective than EW in inhibiting *B. subtilis* subsp. *subtilis* and in reducing total bacterial loads in air- and vacuum-packaged seaweed.**

Key words : *Bacillus subtilis* subsp. *subtilis*, hydrogen peroxide, *Capsosiphon fulvescens*

Introduction

Capsosiphon fulvescens (Mesaengi) is a green algae cultivated on the southern coast of Korea. The cells are less than 10 cm long and 2 cm wide, and are reproduced by biflagellated isogametes released from the bisexual gametophytes(1). In Korea, approximately 1,000 tons (wet weight) of *Capsosiphon fulvescens* are produced per year in South Korea(2). Recently, considerable attention has been given to *Capsosiphon fulvescens* as a functional food. Its market has continued to expand with the increasing consumer preference for seafood. In order to increase the commercial value of seaweed as a food, there has been an effort to prolong the shelf-life of seaweed. Heat-resistant spore-forming bacteria found in seaweed, such as Bacillus strains, deteriorate the quality of seaweed products by breaking down carbohydrates, lipids, and proteins(3). The spoilage by microorganisms is mainly responsible for the softening and relatively short shelf-life of seafood(4). These problems have caused increased interests for disinfectants such as chlorine, hydrogen peroxide, organic acids, and ozone to reduce bacterial loads and inhibit microbial spoilage reactions on many different types of foods, including milk, drinking water,

†Corresponding author. E-mail : jbm@chonnam.ac.kr,
Phone : 82-61-659-3414, Fax : 82-61-659-3419

eggs, vegetables, seafood, and meat(5, 6, 7, 8). Hydrogen peroxide is a recognized bacteriocide and sporicide, and hydrogen peroxide readily reacts with metals such as reduced copper or iron, and forms a powerful oxidant, hydroxyl free radical, which can attack membrane lipids, DNA, and essential cell components(7). In the Fenton reaction, superoxide radical ($O_2^-\cdot$) is produced by a one electron reduction of molecular O_2 via transition metals (Fe, Cu), which are excellent catalysts of O_2 reduction and have unpaired electrons. Superoxide radical reduces both Fe^{3+} and Cu^{2+}, and a hydroxyl radical is generated from the reaction of Fe^{2+} or Cu^{+} with hydrogen peroxide(9).

1. $Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^-\cdot$
2. $Fe^{3+} + O_2^-\cdot \rightarrow Fe^{2+} + O_2$
3. $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO + HO\cdot$

Hydroxyl radicals are extremely reactive and oxidize almost anything, except ozone, due to their high standard electrode potential of +2.3 V (the standard potential of oxygen is ca. +0.8 V), which is second only to fluorine (+3.0 V) (10). All respiring cells normally have defenses against free radicals. These defenses consist of catalases and peroxide dismutases, which inactivate hydrogen peroxide, and superoxide dismutase, which scavenges superoxide radicals thereby preventing formation of hydroxy free radicals however, this defense process can be overwhelmed by high concentrations (3 to 6%) of hydrogen peroxide(7). Recently, acidic electrolyzed water and neutral electrolyzed water were reported to be effective against *Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus cereus*, and *Listeria monocytogenes* as sanitizers(5, 9). Our study evaluated the effectiveness of electrolyzed water (EW), NaOCl, and hydrogen peroxide against *Bacillus subtilis* subsp. *subtilis* and the total viable microorganisms found in *Capsosiphon fulvescens*.

Materials and Methods

Sample

We collected the *Capsosiphon fulvescens* specimens from Janghung, a county located on the southwest coast of Korea, during January 2007.

Biocide solutions The electrolyzed water (EW) was generated using a ROX-20TA EO water generator (Hoshizaki Electric Co. Ltd., Toyoake, Aichi, Japan). The NaOCl and hydrogen peroxide were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The EW contained 50 ppm of available chlorine. The NaOCl (50 ppm) and hydrogen peroxide (50 ppm) were prepared for the experiments.

Measurements of antimicrobial activity Each biocide

(50 ppm) in 10 mL of marine broth was inoculated with aliquots of individual cultures sufficient to produce initial counts of 10^7 cfu/mL. The marine broths were incubated at 37℃ for 24 hr. Each sample was tested in triplicate. After the 24 hr period, optical density was used to determine the antimicrobial effects against *Bacillus subtilis* subsp. *subtilis* by measuring the absorbance at 600 nm using a spectrophotometer (Mecasys Co., Ltd., Korea).

Microbiological analysis

To begin, each frozen sample (200 g) was immersed in 2 L of the test biocide [EW (50 ppm of free chlorine), hydrogen peroxide (50 ppm), and NaOCl (50 ppm)] at 25℃ for 150 min, without shaking. After being treated with the biocide solutions, the samples were washed with 2 L of 0.85 % sterile NaCl. Samples (200 g) were stored at 4℃ for 30 days after being packaged using two methods: air-packing with a volume of about 1100 cm^3 and the vacuum-packing (Magic Seal, Seoul, Korea) at the vacuum of 74 cm/Hg. Ten grams of *Capsosiphon fulvescens* were homogenized using a homogenizer (Omni Macro Homogenizer, Omni international, CT, USA) for 30 sec in 90 mL of PBS (pH 7.2), and then plated on marine agar (Difco Laboratories, Detroit, MI). The plates were incubated at 37℃ for 48 hr in triplicate prior to counting the microorganisms.

Bacterial identification

In order to identify a bacterium based on its 16S ribosomal gene sequence, genomic DNA was extracted with an Accuprep Genomic DNA extraction kit according to the manufacturer's protocol, using a bacterium grown overnight on MA(11). DNA amplification was carried out in the following manner: 5 min of pre-soaking followed by 30 cycles at 94℃, 30 sec at 55℃ for denaturing, 30 sec at 72℃ for annealing, 40 sec for extension, and finishing with an incubation at 72℃ for 5 min using the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3')(13). The PCR products were purified with a Wizard PCR Preps DNA Purification System (Promega) according to the manufacturer's instructions,

and sequenced using a BigDyeTM Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and a model 310 automatic sequencer (Applied Biosystems). The closest known relatives of the new isolates were determined by performing sequence database searches, and the sequences of closely related strains were retrieved from GenBank or the Ribosomal Database Project (RDP) libraries. Nucleotide (NT) sequence similarities were calculated using the PHYDIT program(12,13).

Texture analysis

After washing the *Capsosiphon fulvescens* with seawater or distilled water, the samples were stored at 20℃ and -80℃ for 50 days. They were then placed in a holder that consisted of a 50 mL centrifuge tube without a bottom and containing a cap, to support the *Capsosiphon fulvescens*. The hardness of the samples was measured in triplicate using a rheometer (Sun Scientific Co. LTD, Japan).

Statistic analysis

Statistical analyses were performed using the SPSS Version 9.0 for Windows (SPSS Inc., Chicago, IL, U.S.A.). ANOVA tests were conducted to determine the effects of the different test biocides on antimicrobial activity. Statistical significance was determined at the level of $p<0.05$.

Results and Discussion

The antimicrobial activities of EW, NaOCl, and hydrogen peroxide against *Bacillus subtilis* subsp. *subtilis* in broth culture are shown in Fig. 1, where the initial population of *Bacillus subtilis* subsp. *subtilis* was 10^7 cfu/mL. After incubation at 37℃ for 24 hr, the antimicrobial activities of the hydrogen peroxide (50 ppm) and NaOCl (50 ppm) were significantly higher than the EW (50 ppm) ($p<0.05$). There was no significant difference between the hydrogen peroxide (50 ppm) and NaOCl (50 ppm) ($p<0.05$) in inhibiting the growth of the Bacillus strain.

After storage at 4℃ for 30 days without biocide treatment, there were increases of 3.1 and 4.2 log cfu/g in the air and vacuum-packaged samples, respectively, from the initial total viable counts of the control sample (2.5 log cfu/g) (Fig. 2). From the bacterial identification analysis, *Bacillus subtilis* subsp. *subtilis* was found to exist in large numbers on *Capsosiphon fulvescens* (90% of the total viable bacteria) after being stored at 4℃ for 30 days. Only three species were identified, namely *Achromobacter insolitus, Brevundimonas nasdae*, and *Microbacterium lacticum* (10% of the total bacterial population), during the 30-day storage period. This result suggests that the spore-forming bacteria, *Bacillus subtilis* subsp. *subtilis*, is the fittest survivor in aerobic or anaerobic packaging condition, and is considered to be the primary target microorganism for the quality control of seaweed during long-term storage.

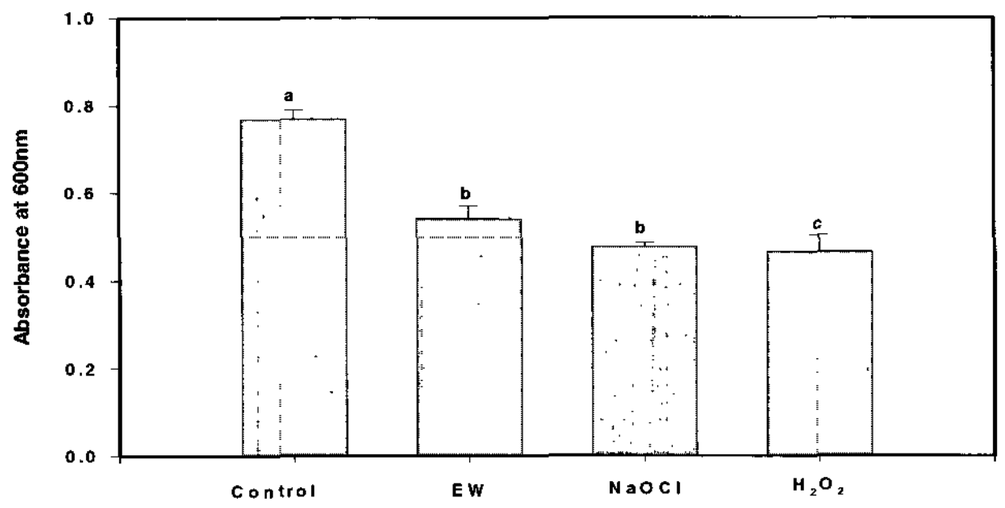

Fig. 1. Effects of electrolyzed water (EW), hydrogen peroxide, and sodium hypochlorite for killing *Bacillus subtilis* subsp. *subtilis*. Each biocide (50 ppm) in 10 mL of marine broth was inoculated with aliquots of individual cultures sufficient to produce initial counts of 10^7 cfu/mL. The marine broths were incubated for 24 hr at 37℃. Each sample was tested in triplicate.

EW, Electrolyzed water; Control, No biocide treatment. Different superscripts (a, b, and c) show significantly different degrees of growth inhibition of *Bacillus subtilis* subsp. *subtilis* by each test biocide ($p<0.05$).

The bactericidal action of hydrogen peroxide was compared to NaOCl and EW by counting the total viable cells of *Capsosiphon fulvescens* in the air and vacuum packages (Fig. 2). The hydrogen peroxide was found to be the most effective in inhibiting the growth of total viable microorganisms during the 30 days at 4 ℃. After 30 days of storage at 4℃, using the air- and vacuum-packaging, the total viable population was reduced by 1.8±0.4 log cfu/g under the 50 ppm treatment of hydrogen peroxide, and was reduced by 1.1±0.2 log cfu/g under the 50 ppm treatment of NaOCl, while EW showed an increase of 1.7±0.5 log cfu/g in total viable counts from the initial loads of the control. There was no significant difference in the reduction of total viable counts between the air and vacuum-packaging methods with the hydrogen peroxide and NaOCl treatments ($p<0.05$). Venkitanarayanan et al.(14) reported that EW showed high antimicrobial activity against planktonic cells. Free chlorine in the EW reacted with organic materials such as amino acids and proteins, and reduced the antimicrobial activity(15). Hydrogen peroxide has shown high antimicrobial activity against *E. coli, S. aureus, S. hyicus, E. faecalis*, and *E. faecium* due to the high penetrability of the free radicals accessing hidden

microorganisms(16, 17, 18). Hydrogen peroxide can be easily decomposed into hydroxy radicals by homolytic fission that results in lethal DNA disruption in *Bacillus subtilus*(19). It can be converted into hydroxyl radicals, which have reactivity second only to fluorine, and can be mixed with water in any proportion(7) to reduce microbial levels in processing lines(8). Lillard and Thomson(20) reported that 6,600 ppm, or higher, of hydrogen peroxide reduced aerobic organisms by 95 to 99.5% in chiller water.

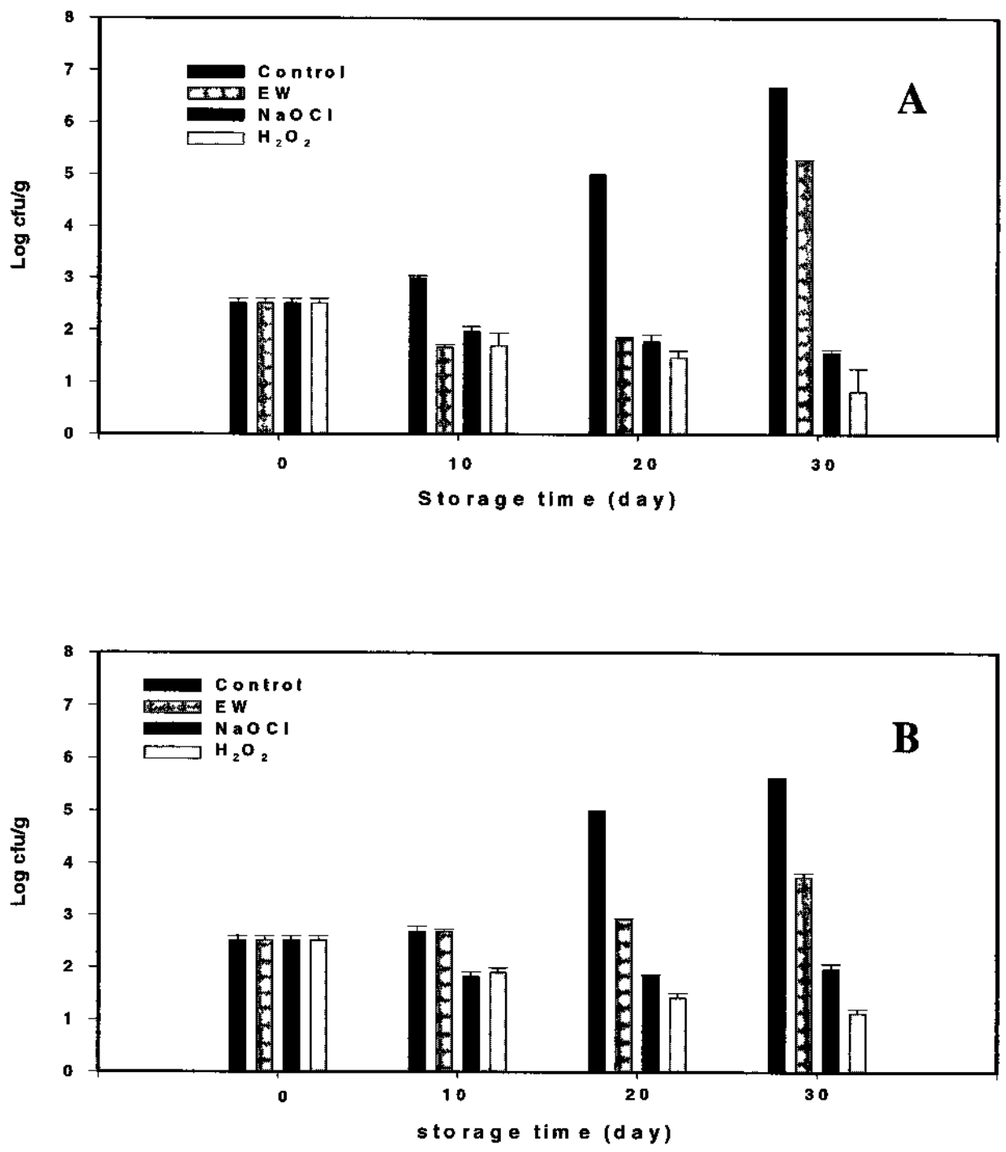

Fig. 2. Effects of electrolyzed water (EW), hydrogen peroxide, and sodium hypochlorite on the reduction of total viable counts in *Capsosiphon fulvescens* packaged under vacuum condition (A) and under air-packaged condition (B).

After being treated with 50 ppm of each test solution, the samples were homogenized, plated on marine agar, and incubated at 37℃ for 48 h in triplicate prior to counting. EW, Electrolyzed water; Control, No biocide treatment.

The hardness of the samples stored at 4℃ for 30 days was measured following the treatments of 50 ppm hydrogen peroxide, NaOCl, and EW (Fig. 3). Hydrogen peroxide and NaOCl showed 1.6±0.1-fold decreases in hardness from their initial hardness (7.9 x106 dyne/cm^2), while the samples treated with distilled water and EW had a 2.1±0.1-fold decrease in hardness from their initial hardness (7.9x10^6 dyne/cm^2) after storage at 4℃ for 20 days, indicating that hydrogen peroxide and NaOCl were more effective than EW at retarding the softening of seaweed fibers by inhibiting the growth of Bacillus strains.

In conclusion, *Bacillus subtilis subsp. subtilis* was found to exist in large numbers on *Capsosiphon fulvescens* during a 30-day storage period. In our examination of antimicrobial activity against planktonic and attached *Bacillus subtilis* subsp. *subtilis* on *Capsosiphon fulvescens*, we found that hydrogen peroxide (50 ppm) and NaOCl (50 ppm) were more effective than electrolyzed water (EW, 50 ppm) in inhibiting *Bacillus subtilis* subsp. *subtilis* and reducing total bacterial loads in air and vacuum-packaged samples. Utilizing hydrogen peroxide and NaOCl as biocides offers a safe and effective means for the decontamination of seaweed, as well as against the spoilage caused by the microorganisms primarily responsible for the softening and relatively short shelf-life of seafood.

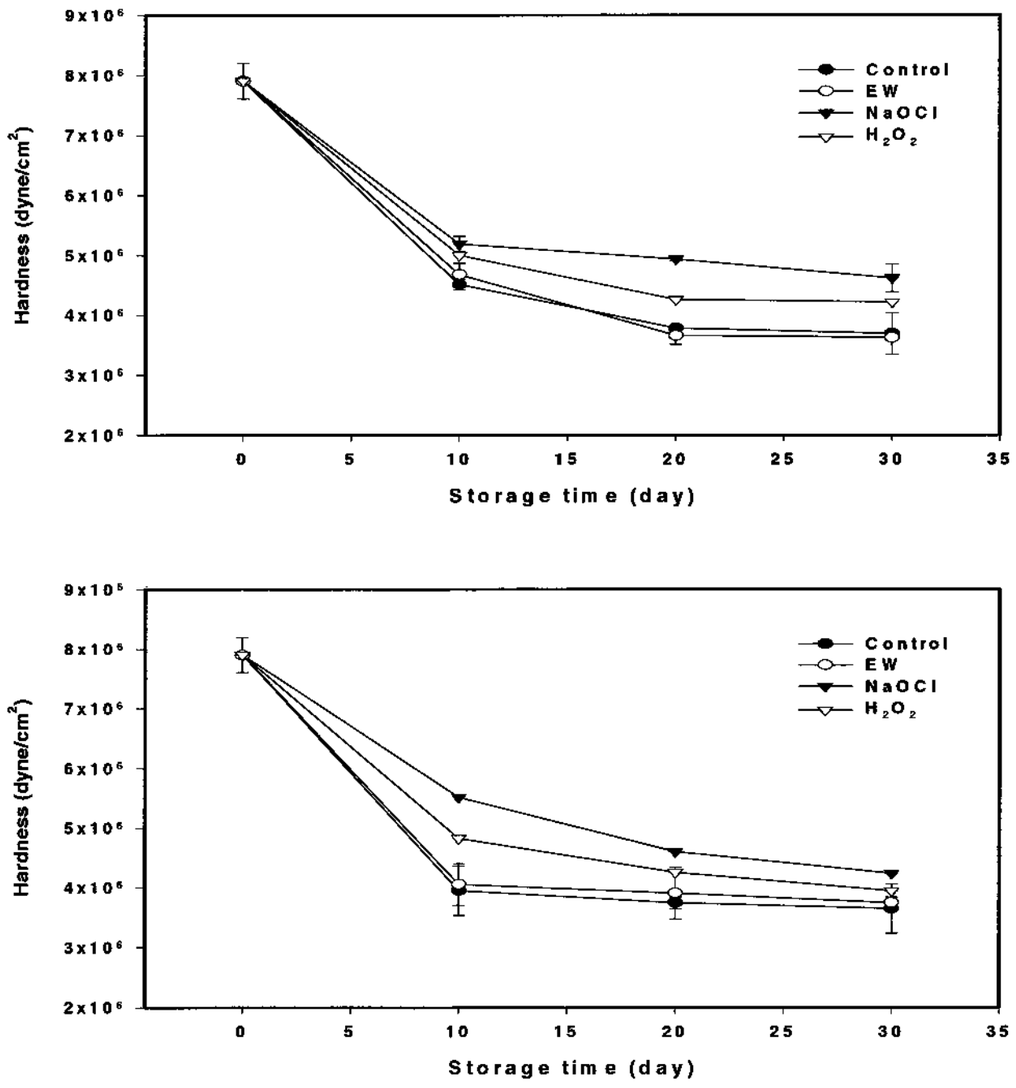

Fig. 3. The changes in texture (hardness) of *Capsosiphon fulvescens* in vacuum-packaged samples (A) and in air-packaged samples (B). Samples were placed in a holder and hardness was measured in triplicate using a rheometer.

요 약

Bacillus subtilis subsp. *subtilis*는 녹조류인 매생이에서 분리되는 총 생균주 중에서 90%를 차지하고 있다. *Bacillus subtilis* subsp. *subtilis*에 대한 항미생물 활성을 50 ppm 수준에서 과산화수소와 NaOCl의 각각의 처리가 전해수 (50 ppm) 보다 유의적 수준에서 높았다. 매생이를 50 ppm의 과산화수소, NaOCl 그리고 전해수로 처리한 후 4℃에서 30일간 일반포장 또는 진공포장에 의한 저장실험 후 총균수를 측정한 결과 과산화수소와 NaOCl를 처리시에는 초기

균수보다 1.8±0.4 log cfu/g 감소함을 보였고 전해수를 처리시에는 1.7±0.5 log cfu/g의 감소를 보였으며, 포장방법에 의한 총균수의 감소에는 영향이 없었다. 매생이를 50 ppm의 과산화수소, NaOCl 그리고 전해수에 처리한 후 4℃에서 20일간 저장실험 후 조직감 (경도)를 측정한 결과, 과산화수소와 NaOCl를 처리시에는 초기 경도 ($7.9x10^6$ dyne/cm^2)보다 1.6±0.1배 감소하였으나, 전해수 처리는 2.1±0.1배의 경도의 감소를 보였다. 결론적으로, 본 연구에서는 *Bacillus subtilis* subsp. *subtilis* 균주가 일반포장과 진공 포장된 제품에서 우점종으로 나타났고, 매생이의 장기 저온 저장 시 중요관리요인으로 나타났으며, 이를 제어하는데 과산화수소와 NaOCl이 전해수보다 효율성이 높았다.

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