PAPER

EFFICACY OF NEUTRALISED ELECTROLYSED WATER AND MILD HEAT AGAINST FOODBORNE PATHOGENS ISOLATED FROM *FICUS CARICA*

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ABSTRACT

Problems with microorganism toxins in dried fig exports are becoming very important. Chlorine-based sanitizers are effective way of controlling microorganisms, but on the other hand their reaction with natural organic and inorganic matter may potentially form carcinogenic products. Therefore, different sanitizers for the disinfection of food and food contact surfaces are required as an alternative to chlorine-based sanitizers. Some earlier studies revealed that neutralised electrolysed water (NEW) may be a potential substitute for cleaning and sanitizing agents in packaged products. In order to make a contribution to solve toxins problems, the antibacterial and antifungal effect of neutralised electrolysed water (NEW) on the foodborne pathogens were evaluated in this study. Spores of *Aspergillus flavus* and *Penicillium expansum* were isolated from the surface of fig fruits. *Escherichia coli and Bacillus cereus* known to occur on the surface of figs were also evaluated. Vegetative cells and spores of bacterium and fungi were exposed to five different concentrations of NEW (100, 75, 25, 5 and 1%) at three different temperatures (22, 50 and 70°C) for 1, 3 and 5 min. According to the results, at 22°C, 1% neutralised electrolysed water exposure for 1 min effectively decreased the number of vegetative cells of *E. coli* and *B. cereus* by approximately 8.5 log cfu/ml and 6.3 log cfu/ml, respectively. At 50°C, 5% neutralised electrolysed water exposure for 1 min significantly reduced the *A. flavus* and *P. expansum* spore numbers by 5.54 log cfu/ml and 7 log cfu/ml, respectively. With the effect of mild temperature (22-50°C), low chlorine neutralised electrolysed water (9.22 mg/l - 33.85 mg/l available chlorine concentrations) showed a strong antibacterial and antifungal activity against foodborne pathogens. As a conclusion, neutralised electrolysed water can be used widely as a sanitizer in fig enterprises, instead of high cost chlorine based disinfectants.

Keywords: *Aspergillus flavus, Bacillus cereus, Penicillium expansum,* sanitizer

1. INTRODUCTION

Figs (*Ficus carica L.*) are an economically important and highly valued fruit with high contents of fibre, minerals and polyphenols. They are mainly grown in the Aegean region of Turkey. With the yearly production of 275,002 tons, Turkey ranks the first in the World in fig production and exportation (TUIK, 2012). Turkey owns the 26% of the world's fig production and 36% of the world's fig exportation (ÇALIŞKAN and Polat, 2012). Thirty percent of figs produced in Turkey are marketed as fresh figs, while the remaining fruit is consumed as dried figs. Due to the high content of carbohydrate and water activities of semi-dried figs on the tree and fallen figs collected from the soil, figs can be considered a good substrate for mycotoxin formation. Water activities for semi-dried on the tree, fallen figs collected from the soil, figs taken from the drying stage and those from warehouses have been reported as 0.88- 0.94; 0.76-0.87; 0.70-0.80 and 0.69-0.73a_w, respectively (KARBANCIOGLU-GULER and HEPERKAN, 2009). The most common toxigenic fungi reported in Turkish dried figs are *Aspergillus* section *Nigri*, *Fusarium spp.*, and *Aspergillus* section *Flavi Penicillium spp.*; their toxin types are aflatoxin, citrinin, fumonisin, patulin, and ochratoxin A (HEPERKAN, 2006). Figs are usually contaminated with *Escherichia coli, Bacillus cereus* and *Bacillus cereus* spores. The numbers of these microorganisms on dried figs were reported to vary from a few hundreds to thousands per gram of fruit (FRAZIER and WESTHOFF, 1988; AKBAS and OZDEMIR, 2008). Poor storage conditions such as damp environments and high storage temperatures may cause bacterial growth to reach to a level of about 10^7-10^8 cfu/g for dried figs. The main purpose of all methods applied for food preserving is to prevent or limit microbial and enzymatic changes in foods. It was estimated that food-borne diseases cause approximately 76 million illnesses, 325,000 hospitalisations, and 5,000 deaths in the United States each year (MEAD *et al*., 1999).

According to the Environmental Protection Agency (EPA) (2001), chlorine-based sanitizers are effective way of controlling microorganisms, but on the other hand their reaction with natural organic and inorganic matter may potentially form carcinogenic products (FAWELL, 2000; ALLENDE *et al*., 2009). Many of these products may cause cancer and generates reproductive and developmental problems in laboratory animals. Therefore, different sanitizers for the disinfection of food and food contact surfaces are required as an alternative to chlorine-based sanitizers.

Electrolysed water generation technology was initially developed in Russia (MAMADZHANOV *et al*., 1974), and then modified in the 1980s by Japanese food industry researchers (HATI *et al*., 2012). In this technology, by using tap water and salt, system generates two stream simultaneously, one is alkaline and the other is acidic, with the characteristics of cleaning and sanitising solutions, respectively. Researchers evaluated the effectiveness of these solutions as a sanitizing and cleaning agent. Studies revealed that they can be a potential effective substitute for cleaning and sanitizing agents in packaged products (IWASAWA *et al*., 1993; HAYASHIBARA*Et al*., 1994).

The electrolysed water acidic solution is reported to be highly effective in deactivating the *E. coli*, *S. enteritides, S. aureus, C. albicans* and *L. monocytogenes* strains (VENKITANARAYANAN, 1999; ILERI *et al*., 2006). The disinfection mechanism of the acidic electrolysed water can be attributed to several factors, including the destruction of bacterial protective barriers, the increase in membrane permeability, the leakage of cellular inclusions, and the decrease in activities of some key enzymes (ZENG *et al*., 2010). However, the potential application of Strong Acid Electrolyzed Water (SAEW) is limited because of its low pH ($\leq$ 2.7). At this low pH, dissolved Cl_2 gas can be rapidly lost due to volatilization, decreasing the bactericidal activity of the solution by the time (LEN *et al*., 2000) and may affect human health and the environment adversely. Moreover, the high

acidity of strong acid electrolyzed water may cause the corrosion of equipment and consequently limit its practical application.
In Japan, strong acid electrolyzed water (pH 2.5±0.2; 20-60 mg/l available chlorine concentrations (ACC)) and slightly acidic electrolysed water (pH 5.0-6.5; 10-30 mg/l ACC) usage in food products is authorised by the Japanese Ministry of Health and Welfare (ZACHARIA *et al*., 2010).
Recently, slightly acidic or neutralised electrolysed water has been of interest, as it minimises human health and safety risks from Cl_2 degassing, reduces corrosion of surfaces, and limits phototoxic side effects (GUENTZEL *et al*., 2008). In spite of these advantages, neutralised electrolysed water *in vitro* inactivation of different food pathogens has not been intensively studied.
Our previous study is among the very few preliminary one aimed to unveil the bactericidal effectiveness of acid electrolysed water against different food spoilage and pathogenic microorganisms (ILERI *et al*., 2006).
In Turkish enterprises, routine fig processing as follows; initially dried figs are washed with salty (3-6% NaCl) high-temperature water (60-80ºC) and then processed further and finally packaged. The presence of salt increases the heat resistance of spores due to reduced water activity leading to spore dehydration (JUNEJA and SOFOS, 2002). While this treatment may not be enough to decrease the microbial population of figs.
Thus it was suggested that neutralised electrolysed water was used instead of salty and high temperature water in fig enterprises to the optimum temperature and the optimum concentration of NEW under laboratory conditions.
Therefore, the effectiveness of NEW on spores of *Aspergillus flavus* and *Penicillium expansum* isolated on the surface of fig fruits, vegetative cells of *Escherichia coli* ATCC 35218 and *Bacillus cereus* ATCC 11778 known to occur on the surface of figs and *Bacillus cereus* ATCC 11778 spores was evaluated under controlled conditions.

2. MATERIALS AND METHODS

2.1. Bacterial cultures and the preparation of inocula

E. coli ATCC 35218 and *B. cereus* ATCC 11778 were plated on Nutrient Agar (NA) and incubated at 37°C for 24 h. after incubation, *E. coli* ATCC 35218 and *B. cereus* ATCC 11778 suspensions were prepared by transferring several colonies to a 10 ml of NaCl solution (0.9%, w/v) with the sterile inoculation loop and vortexed using a thermal mixer. The 1 mL aliquots of undiluted and diluted cultures were transferred to a cuvette, and optical densities of the solutions at 600 nm were measured. Aliquots of the samples were also used to perform serial dilutions. Dilutions were plated on NA plates and incubated at 37°C overnight. Colonies were counted to determine viable bacterial cell counts (cfu/mL) at each dilution ratio, and then a standard curve was prepared by using absorbance and viable cell count.

2.2. Preparation of spore solution of *B. cereus*

B. cereus ATCC 11778 was grown on nutrient agar (Difco, Becton Dickinson, Detroit, MI, USA) containing 500 ppm Bacto Dextrose and 3 ppm manganese sulphate for 7 days at 30°C. After this incubation, sporulation was determined by the Schaeffer-Fulton method (SCHAEFFER and FULTON, 1933). Each agar plate with spores was rinsed twice with approximately 10 ml of sterile-distilled water while each plate was revolved on a

turntable, and the agar surface was gently agitated with a glass spreader to facilitate spore removal or the harvesting of the spores.
The accumulated spore suspensions were washed four times by centrifugation at 4000 rpm for 20 min and re-suspended in sterile NaCl solution (0.9%, w/v). Tween 80 (0.05%, v/v) (Sigma, St. Louis, MO, USA) was added to spore suspensions in order to minimise the clumping of spores and improve the accuracy of spore counts. All transfers, inoculations and harvesting for the preparation of spore suspensions were performed under laminar flow conditions.
The spore counts were enumerated by direct microscopic counting on a Thoma slide. For verification of the number of spores in the suspension, the spore suspension was first heat-shocked at 80°C for 10 min to kill vegetative cells. Next, the number of viable spores was estimated by spread-plating 0.1 ml of spore suspension on nutrient agar at 30°C for 24 h. The spore suspension was stored at 4°C until use (AKBAS and OZDEMİR, 2008).

2.3. Preparation of spore solution of *Aspergillus flavus* and *Penicillium expansum*

Aspergillus flavus was isolated from fig fruits in the laboratory of Adnan Menderes University. *A. flavus* was cultured on potato dextrose agar plates (Merck) at 25°C for one week. After a portion (about 15 ml) of 0.1 M dibasic sodium phosphate adjusted with 0.05 M citric acid monohydrate of pH 7.0 (PC) with 0.005% (v/v) Tween 80 was poured onto the plate, spores were suspended in PC buffer by rubbing gently with a glass rod. Clumped spores in PC buffer were scattered by sonication for 2 min. The suspension was filtered through eight layers of sterile cheese cloth to remove hyphae. The spore concentration of the suspension was estimated with a haemocytometer. The suspension was diluted with PC buffer to obtain a concentration of 107 cells per ml (FUJİKAWA and ITOH, 1996).
Penicillium expansum was isolated and identified from fig fruits at the microbiology laboratory of Adnan Menderes University. Spore suspensions were prepared on Petri dishes for 1 week with PDA and 50 mg/l of streptomycin. After one week of incubation at 25°C, spores were collected and suspended in sterile Ringer's solution. After filtering through eight layers of sterile cheese-cloth, spores were counted and brought to a final concentration of 107 cell per ml (SPADARO *et al*., 2002).

2.4. Preparation of treatment solutions

NEW was obtained by electrolysis of a mixture of NaCl (20 g/l) and tap water using STEL-10H-120-01 generator (STEL - 10H- 120-01, Russia) at 40.0 V, 9.0 A and produced at a rate of 250 ml/22 sec. A cylindrical electrochemical cell for processing solutions comprised an inner, hollow, tubular anode, an outer, cylindrical cathode, and a permeable, tubular, ceramic diaphragm that was arranged between the anode and cathode and divided the inter-electrode space into anode and cathode chambers so that a working section of the cell was formed. There was a hole connecting the internal chamber with the channel for the liquid withdrawal. The internal chamber was connected by the annular channel to the horizontal channel used for feeding liquid subjected to the treatment.
The NEW dilutions were prepared by using sterile tap water at rates of 100% (undiluted), 75%, 25%, 5% and 1%, while deionised water (DW) alone was used as control. Analytical indices (ORR, pH and ACC) of the treated solutions were measured immediately after NEW preparation and before each bactericidal and fungicidal experiment. The pH was measured with a pH meter (HI 2211-02, HANNA, USA), and ORP was measured with an ORP meter (HI98120, HANNA, USA). The pH meter was calibrated using commercial

standard buffers at pH 4.0 and 7.0 (Merck Ltd., Germany). The ACC was measured on the basis of the iodometric method reported by DYCHDALA (1983).

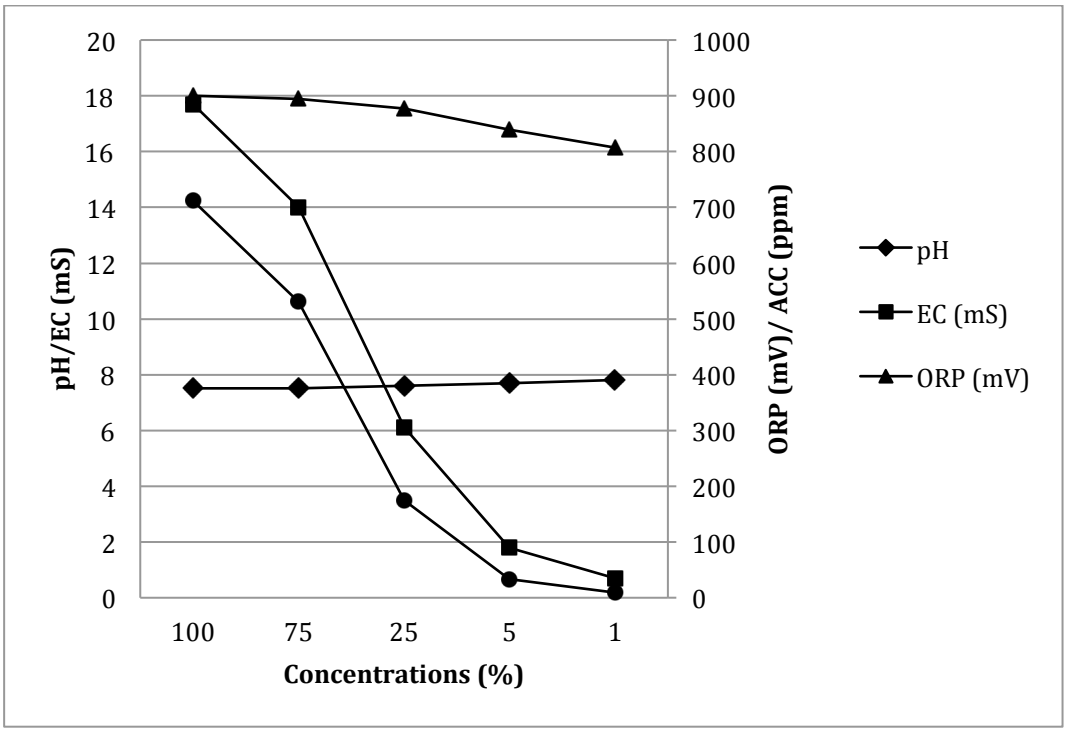
2.5. *In vitro* inactivation of microorganisms by NEW

In vitro inactivation experiments for their disinfection potential against vegetative cells of *E. coli* ATCC 35218, *B. cereus* ATCC 11778 and spores of *A. flavus*, *P. expensum*, and *B. cereus* were carried out at 22, 50 and 70°C for 1, 3, and 5 min. The bacteria and spore suspensions were prepared as described above. Different concentrations of the NEW solutions were prepared with sterile tap water. Before use, each NEW was diluted and placed in a water bath at 22, 50 and 70°C to reach the desired temperature before inoculation. One millilitre of each vegetative cell and spore suspension was separately added to 9 mL of different concentrations of NEW (1, 5, 25, 75, and 100%, v/v) and sterile DW. After inoculation, 1 mL aliquots were periodically collected at the end of incubation time (1, 3, and 5 min) at 22, 50 and 70°C, and transferred into the membrane filter funnel containing sterile DW (50 ml). The samples were drawn completely through the filter. The funnel was rinsed with sterile DW (150 ml). The membrane filter was removed from the funnel and placed into the prepared Petri dish. Agar plates were incubated at the proper temperature (bacterium and fungi, 35±2 and 30°C, respectively) and for the appropriate time period (bacterium and fungi, 24 and 72 h, respectively).

3. RESULTS AND DISCUSSIONS

3.1. Physicochemical properties of tested solutions

One of the first applications of electrolysed water was the sterilisation of medical instruments in hospitals; it has since been used in the medical, dental, agriculture, dairy and food industries. Based on the literature review carried out as part of this study, it was found that applications in the food industry have been the most frequently researched.
Applications of electrolysed sanitising solutions for microbial inactivation in fresh lettuce have been reported by PARK *et al*. (2001), KOSEKI *et al*. (2003), DELAQUIS *et al*. (2004) and ONGENG *et al*. (2006). It was also used for the disinfection of tomatoes (Bari *et al*., 2003), spinach (RAHMAN *et al*., 2010), cucumbers (KOSEKI *et al*., 2004) and strawberries (KOSEKÎ *et al*., 2003; UDOMPIJITKUL *et al*., 2007).
To provide ease of use in fig enterprises, tap water was used for the preparation of different concentrations of NEW. The ORP, pH and ACC for the treated solutions are shown in Figure 1. The pH, ORP and ACC for five different concentrations of NEW solution (100, 75, 25, 5 and 1%) used in this study were pH 7.45-7.84, 900-807 mV and 712.6-9.2 mg/l, respectively.
Differences between the pH and ORP of different concentrations of EAS were very small, but the difference between ACC was significant. The DW was used as a control solution in this study; it had a pH of 7.58, an ORP of 353 mV, and no available chlorine was detected.



Concentrations (%)	100	75	25	5	1
pH	7,5	7,5	7,6	7,7	7,8
EC (mS)	17,7	14	6,12	1,8	0,7
ORP (mV)	900	894	877	840	807
ACC (ppm)	712,6	530,2	174,7	33,9	9,2

Figure 1: Physicochemical properties of tested solutions.

3.2. Antimicrobial effects of different dilutions of NEW on pure cultures of *E. coli, B. cereus and spores of B. cereus, A. flavus and P. expansum*

For vegetative cells, very rapid killing was observed. At all of the concentrations tested, NEW reduced bacterial numbers to the detection limit shown below within 1 in at 22°C (Figs. 2, 3), corresponding to a $\log_{10}$ reduction factor of 8.5 and 6.3 (within 1 min) for *E. coli* and *B. cereus*, respectively.

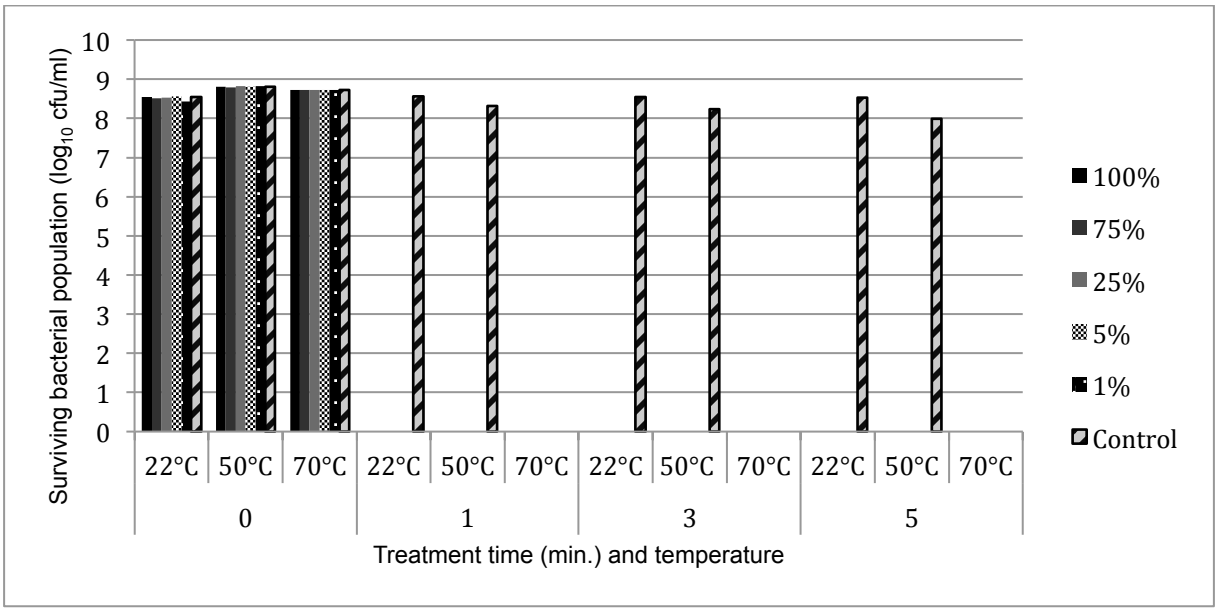


Figure 2: The effectiveness of different concentrations of NEW against *E.coli* ATCC 35218 *vs* treatment time and temperature.

At 50°C, the 1 min treatment of control group of *B. cereus* achieved the 0.6 $\log_{10}$ cfu/ml reduction of their population. However, under the same conditions, a pure culture of *B. cereus* vegetative cells using 1% NEW (available chlorine of 9.22 mg/l) was reduced by 6.6 $\log_{10}$ cfu/ml (8.06 $\log_{10}$ cfu/ml to 1.5 $\log_{10}$ cfu/ml). The decrease in the control group depends on temperature and was found to be 1.6 $\log_{10}$ cfu/ml at the same temperature for 5 min (Fig. 3).

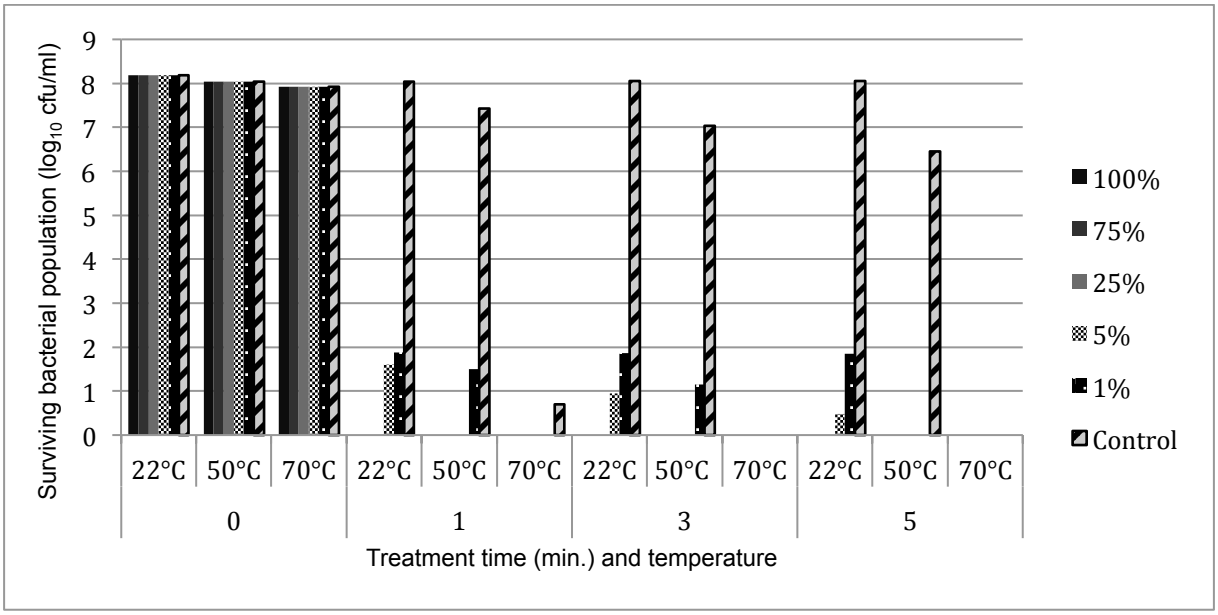


Figure 3: The effectiveness of different concentrations of NEW against *B. cereus* ATCC 11778 *vs* treatment time and temperature.

At 70°C, a reduction of 7.2 and 7.92 $\log_{10}$ cfu/ml in the control group was observed for 1 and 5 min, respectively (Fig. 3). ISSA-ZACHARIA *et al*. (2010) reported that SAEW (pH 5.6, 23 mg/l ACC; and 940 mV ORP) effectively reduced the populations of *E. coli, S.aureus* and *Salmonella spp*. by 5.1, 4.8, and 5.2 $\log_{10}$ cfu/ml, respectively. KIM *et al*. (2000) observed that *B. cereus* was more resistant to treatment than *E. coli* O157:H7 and only 3 log reductions were achieved after 10 s of ROX EO water treatment. Kiura *et al*. (2002) showed that reduction of the population of *B. subtilis* by 3.5 $\log_{10}$ cfu/ml was achieved by 5 min of acid electrolysed water (AEW) treatment (pH 2.32, 4.95 mg/l ACC).
The effect of ozonation was investigated to reduce *E. coli, B. cereus* and *B. cereus* spores in dried figs. *B. cereus* spores, *E. coli* and *B. cereus* counts were decreased by 3.5 log numbers at 1.0 ppm ozone concentration for 360 min ozone treatment (AKBAS and OZDEMIR, 2008).
The effectiveness of different concentrations of NEW was evaluated against spores of *A. flavus* and *P. expansum* using varying treatment times and temperatures *in vitro* (Figs. 4 and 5). It was observed that spores were more highly resistant to the activity of NEW than vegetative cells. At 22°C, the treatment of spores of *A. flavus* for 1 min using NEW (25%, 174.7 mg/l) resulted in a reduction of 5.83 $\log_{10}$ cfu/ml. Under the same conditions, the reduction of spores of *P. expansum* was 6.04 $\log_{10}$ cfu/ ml. Antimicrobial effectiveness of NEW associated with contact temperature increased up to 50°C, but the control group was not affected by this increase in temperature. At 50°C, the numbers of spores of *A. flavus* and *P. expansum* were reduced by more than 5.54-7 $\log_{10}$ cfu/ml within 1 min of exposure to NEW (33.9 mg/l ACC) (Figs. 4 and 5).

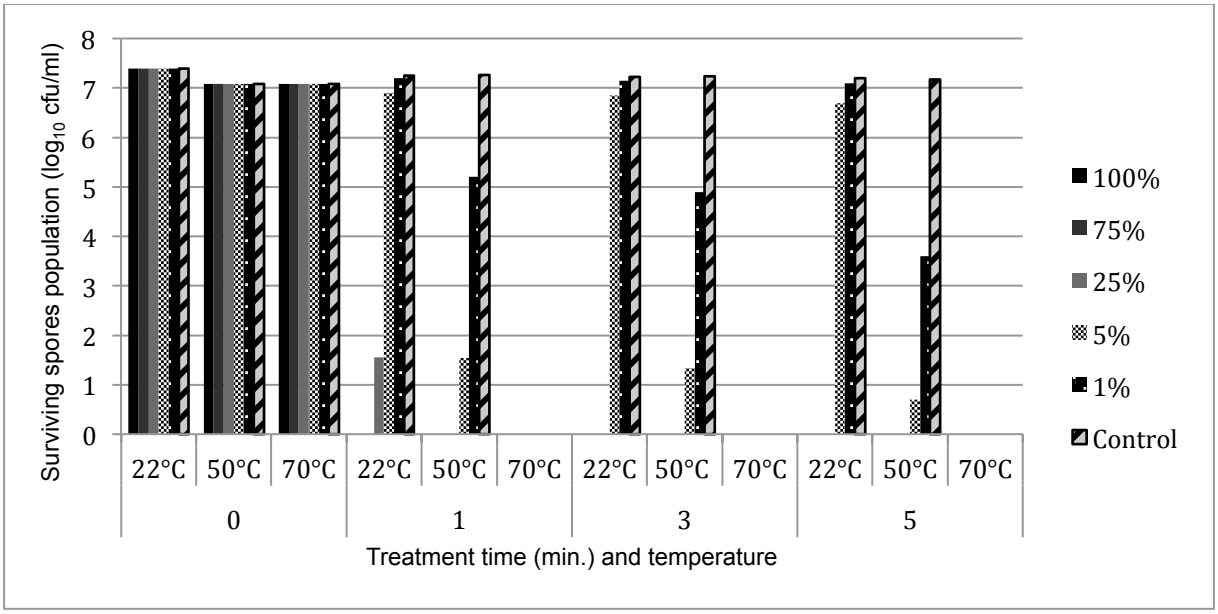


Figure 4: The effectiveness of different concentrations of NEW against spores of *A. flavus vs* treatment time and temperature.

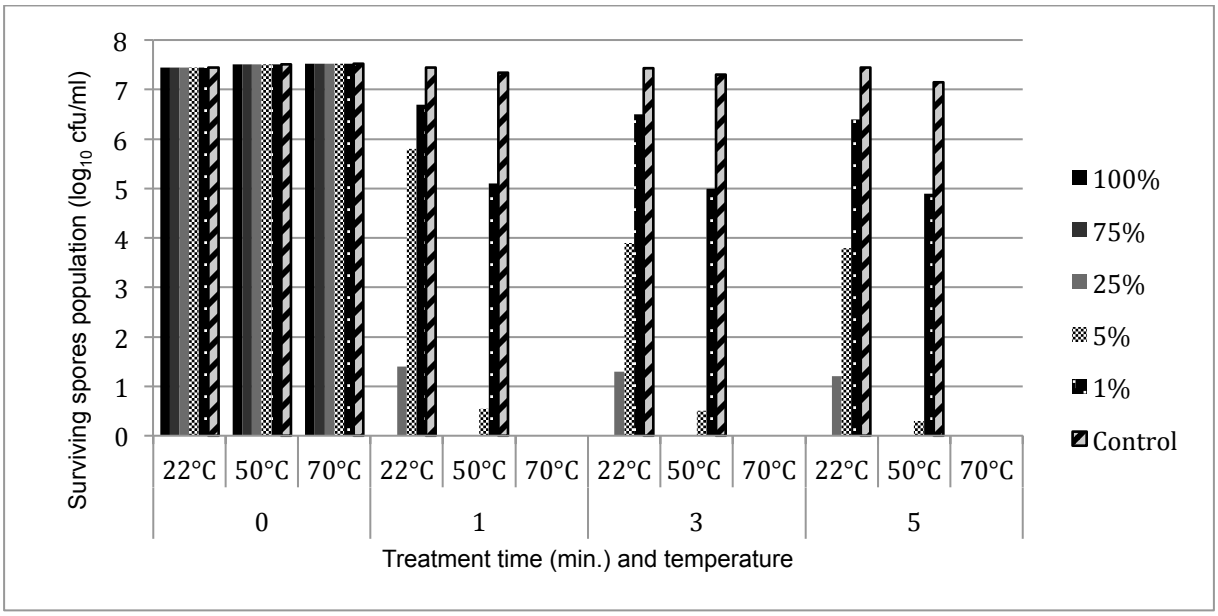


Figure 5: The effectiveness of different concentrations of NEW against spores of *P. expansum vs* treatment time and temperature.

At 70°C, populations of the control groups decreased to undetectable levels. Therefore, the effectiveness of NEW was not determined at this temperature (Figs. 4 and 5). Chlorine at levels of 100 to 200 ppm is currently recommended for the control of postharvest pathogen spores in dump tanks and other recirculating water systems (WILLET *et al*., 1989). However, the use of chlorine has disadvantages, such as the corrosion of metal equipment, reliance on manual monitoring of chlorine concentrations, sensitivity to organic load, effectiveness within a narrow pH range, and the formation of harmful chlorinated by-products (ROBERTS and REYMOND, 1994).

EAW is an alternative to chlorine and chlorine compounds; it has the lowest amount of free chlorine and a very wide pH range. Therefore, it has been reported that EAW can be effectively used for the control of postharvest pathogen spores (OKULL and LABORDE, 2004; AL-HAQ and SUGİYAMA, 2004; KOSEKI *et al*., 2004). In their studies on a laboratory scale, Audenaert *et al*. (2012) demonstrated that electrolysed oxidising water (EOW) has the potential to control *Fusarium* spp. in wheat grains.

It was observed that *B. cereus* endospores were substantially more resistant to the activity of NEW and temperature than fungi spores. Control groups of *B. cereus* spores were not affected by high temperature (70°C), but reduction of *B. cereus* spores exposed to NEW was increased at the same temperature. At 22°C, decreases in viable spores were initially rapid with a more than 5 $\log_{10}$ cfu/ml reduction observed after 1 min of exposure to undiluted NEW (100%). Under the same conditions, log reduction of 4.77 $\log_{10}$ cfu/mL was obtained when the concentration of NEW was decreased from 100% to 75%.

A longer exposure time was more effective in reducing the spore germination. After 3 min of exposure to 100, 75 and 25%, log reductions of 6.26 log10 cfu/ml, 4.84 $\log_{10}$ cfu/ml and 4.3 $\log_{10}$ cfu/ml, respectively, were observed.

At 22°C and 100% NEW, the number of viable spores dropped below the experimental detection limit within 5 min, which corresponds to the log reduction factor of 7.04. After 5 min of exposure to 75% NEW, 1.86 $\log_{10}$ cfu/ml viable spores were still observed (Fig. 6). It was determined that NEW, which has a low concentration and low content of ACC, was

more effective against spores of *B. cereus* at high temperatures. At 70°C, 5 $\log_{10}$ cfu/ml reductions were achieved after 1 min of 5% NEW treatment (Fig. 6).
Robinson *et al*. (2010) reported that, at the concentration of 99%, analytes produced a log reduction factor of greater than five in viable *B. atrophaeus* endospores within 90 s, and at concentrations of 10% or lower, the sporicidal effect was not statistically significant.

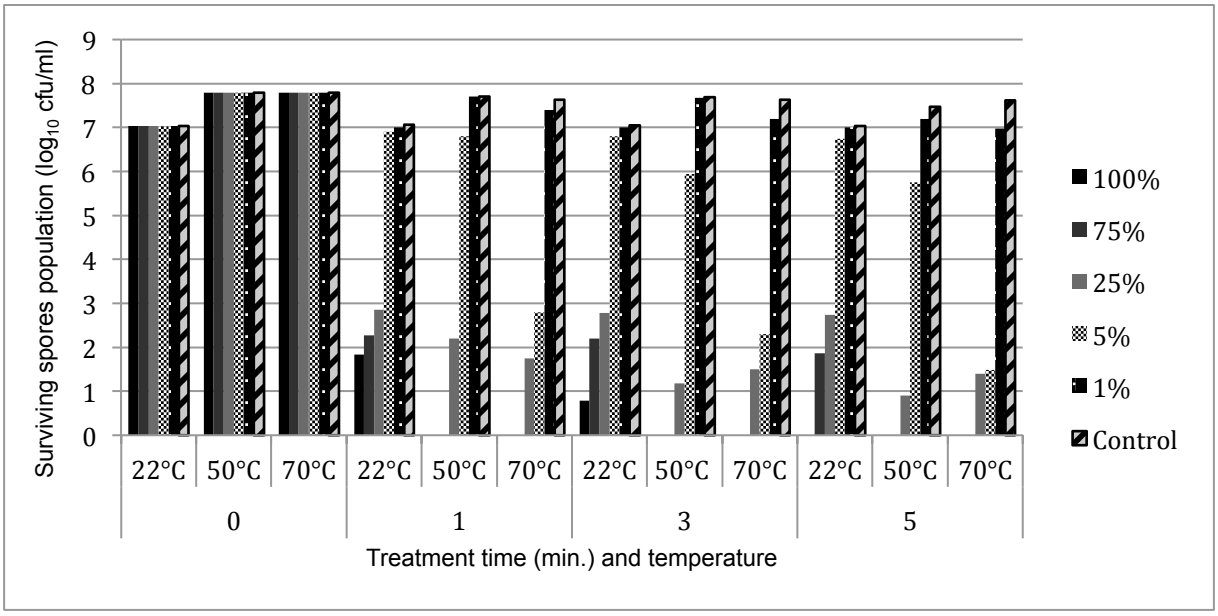


Figure 6: The effectiveness of different concentrations of NEW against spores of *B. cereus* ATCC 11778 *vs* treatment time and temperature.

Physicochemical properties and bactericidal activity of NEW and AEW under four different storage conditions were investigated by Cui *et al*. (2009). They reported that NEW with a near-neutral pH (pH 6.5) and low ORP value (550 mV) had a bactericidal activity (pH <3.0, ORP > 1000 mV) similar to AEW. Different researchers have reported similar results to those from the study of CUI *et al*. (2009) (MARAIS and BROZEL, 1999; KIURA *et al*., 2002; ROBINSON *et al*., 2010). They observed that available chlorine is one of the main contributing factors for the antimicrobial activity. Our results also indicated that the concentration of free chlorine in EOW was more substantial for the microbial growth reduction than pH.

4. CONCLUSIONS

The NEW solution was synthesised as the result of the electrochemical activation. The solution of high reaction ability is in the meta-stable state and contains strong oxidants such as ClO_2, HClO, ClO^-, Cl^-, HO_2, O_3, H_2O_2, H_3O^+, Cl_2O etc. It should be noted that somatic cells of animals and humans have a system of enzymatic protection against the action of the peroxidation factors mentioned. Microbial cells, especially anaerobic ones, are deprived of this protection. This fact determines the high selectivity of the NEW action on the micro flora. The findings of this study demonstrated that the bactericidal efficacy was retained against vegetative cells at dilutions as low as 1% and against bacteria and fungi spores at dilutions as low as 5% NEW. Also, 5% NEW with a pH of 7.7, ACC of 33.9 mg/l

and ORP value of 840 mV can effectively inactivate pure cultures of spores of *B. cereus* and fungi *in vitro*. In addition, as a result of being non-corrosive, more stable for storage, inexpensive, and posing less potential health hazards to workers due to a lack of Cl_2 degassing, 5% NEW has more potential for application in fig enterprises than AEW (acidic and includes high available chlorine) and chlorine-based disinfectants, which have high associated costs.

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