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DEPARTMENT OF AGRICULTURAL AND BIOLOGICAL ENGINEERING

DECONTAMINATION OF *ESCHERICHIA COLI* K12 NSR ON STRAWBERRIES BY ELECTROLYZED OXIDIZING WATER

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ABSTRACT

Food safety is extremely critical in order to ensure a customer's well-being when consuming the target product. Fresh produce has an especially crucial focus due to the fact that they are picked freshly at a farm, so they could easily get contaminated from the soil during the harvesting process. Therefore, they must be handled and processed with extra precautions in place in order to not compromise customer safety and the overall quality. Electrolyzed oxidizing water, also known as EO water, has been used as a novel method to clean and decontaminate fresh produce. EO water is produced by the electrolysis of dilute sodium chloride solution, and it can kill microorganisms effectively. This project focuses on the inactivation of *Escherichia coli* K12 nalidixic acid and streptomycin sulfate (NSR) resistant strain on strawberries. From the results of preliminary studies and trials, the optimal condition to treat strawberries was determined to be 80% EO water at 32°C for 30 min in order to reduce the population of *E. coli* K12 NSR by 7-log. In addition, the quality study on colors of the strawberries before and after treatment was performed as well as the shelf life study of untreated and treated samples at both room (~22°C) and refrigerated (~4°C) temperatures. The quality test, measured by colorimeter, showed that there was no significant difference in the appearance of samples after the treatment. The shelf life study for the 7-day period at room temperature demonstrated that the treated strawberries formed less molds and less rotten surfaces compared to the untreated ones, and the samples stored in the refrigerator for 14 days showed that the quality of treated samples could stay excellent for two weeks without showing mold growths while the untreated samples had signs of mold growths. In conclusion, this study clearly demonstrated that EO water can be used to decontaminate strawberries to assure safety and extend shelf-life.

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Chapter 1

Introduction

Foodborne illness has always been a serious problem in the United States, and the number of incidents has been growing over the years. According to Centers of Disease Control and Prevention, each year approximately 1 in 6 Americans gets sick, including 128,000 hospitalized, and 3,000 died from foodborne diseases (CDC, 2013). The number of fresh produce outbreaks has been increasing each year, which is not only affecting the consumers but the market. According to a report, the 10 biggest foodborne outbreaks are associated with meat and fresh produce (Andrews, 2013).

During the processing steps for fresh produce, the most important first step is to wash it in order to ensure the quality of the produce is good and the surface clean. Electrolyzed oxidizing (EO) water is a novel cleaning and sanitizing method, first developed in Japan, to process fresh produce and achieve decontamination. It is better compared to the traditional thermal or chemical processes, which involve chemical residues, limited effectiveness, discoloration of foods, and high cost; EO water is more cost effective, user and environmentally friendly and could achieve the inactivation of a number of microorganisms (Demirci and Ngadi, 2012). For example, Pangloli and Hung (2013) investigated and compared the effectiveness in microbial reduction of *E. coli* O157:H7 on blueberries by various methods including tap water, ozonated water, commercial product such as FIT® solution, EO water, and bleach (Sodium Hypochlorite). The most reduction in this specific pathogen was achieved with the bleach solution, followed by EO water, FIT® solution, ozonated water, and tap water. FIT solution, EO water, and bleach solutions were the three methods that successfully achieved complete inactivation of *E. coli* O157:H7. Based on the results of the study, EO water and bleach solution could reduce the population

significantly more than the other methods (Pangloli and Hung, 2013). EO water's key properties, such as low pH and high oxidation reduction potential (ORP), were a very important factor for such achievement.

EO water has been recognized as an effective method in sanitizing and disinfection due to its strong antimicrobial characteristics as well as the property of low pH, high oxidation reduction potential and hypochlorous acid (Demirci and Wang, 2013). EO water is produced through electrodialysis, which is a membrane separation process driven by electric current. Figure 1 below is the EO water generator machine (Model ROX 60SA by Hoshizaki Electric Co. Ltd., Japan) that is used to conduct this project.

Two major types of EO water, catholyte and anolyte, are produced from the generator. Catholyte is also known as the alkaline EO water, which is basic and has a pH about 10-11. It is used mainly as a cleaning agent. The other type, anolyte, also known as the acidic EO water, has a typical pH of 2 to 3. The acidic EO water is suitable for microbicidal purposes (Demirci et al., 2011). Even though the acidic EO water has a low pH, it is not corrosive to the skin, animal tissues, or mucous membrane (Demirci and Ngadi, 2012). Figure 1 shows how the generator works to produce EO water.

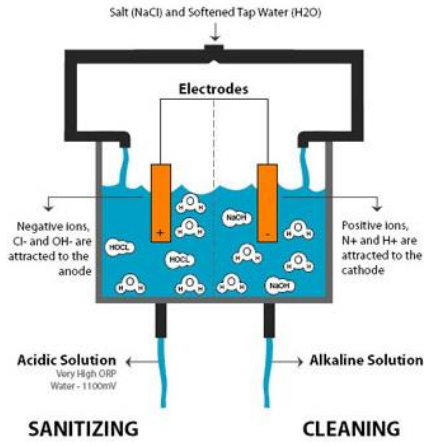

Figure 1. Schematic of EO water generation.

There are a few companies and suppliers in the world that manufacture EO water generators, such as Hoshizaki Inc. from Japan, Aquaox LLC from FL in the United States, and Envirolyte from New Zealand. There is also "neutral EO water" that is commercially available; it is produced from a mix of alkaline solution and the acidic solution, which has a pH close to 7 and an ORP of 700 mV on average. Compared to the acidic EO water, the neutral EO water has a relatively higher pH; therefore, it is less corrosive while still having the disinfecting and cleaning function from its high ORP (Demirci and Wang, 2013). A previous study on using neutral EO water to reduce bacteria on spinach and lettuce was done in 2006 and had promising reduction results in microbial population. The effectiveness of neutral EO water was evaluated using pure cultures of *Escherichia coli, Salmonella typhimurium, Staphylococcus aureus, Listeria monocytogenes*, and *Enterococcus faecalis*, which all yielded 100% inactivation, about 6.1-6.7 log CFU/ml (Guentzel et al., 2006).

Prior to this project, other research studies had been done to investigate the decontamination ability of EO water. For example, EO water was used to treat *E. coli* O157:H7 and *Listeria monocytogenes* on raw salmon. The results from response surface analysis demonstrated maximum log reductions of 1.33 $\log_{10}$ CFU/g (95.3%) for *E. coli* O157:H7 and 1.09 $\log_{10}$ CFU/g (91.9%) for *L. monocytogenes* (Ozer and Demirci, 2006). The optimal condition of *E. coli* O157:H7 found from the study was treatment of alkaline EO water for 17.5 min and followed by acidic EO water for 30 min at 22°C and alkaline EO water for 30 min followed by acidic EO water for 30 min at 28°C for *Listeria monocytogenes* (Ozer and Demirci, 2006). The treatment method used in the study was to hang raw salmon fillets with a hook in EO water solution for treatment and a magnetic agitator was placed in the setup to ensure the inoculum was evenly distributed, which could effectively decrease the variation and increase consistency.

Moreover, alfalfa seeds and sprouts were both washed and treated by EO water and the results indicated that the reduction was between 38.2% and 97.1% (0.22–1.56 $\log_{10}$ CFU/g) in the

bacterial load of treated seeds and between 91.1% and 99.8% (1.05–2.72 $\log_{10}$ CFU/g) for the sprouts (Sharma and Demirci, 2003). The alfalfa seeds and the sprouts were both treated with one liter of acidic EO water by continuous agitation. It is interesting that the study found that higher amperage level of the EO water generator, corresponding to higher concentration of free and total chlorine, was more effective in decontaminating both alfalfa seeds and sprouts, yet an increase of treatment contact time with EO water did not yield a greater reduction in population. Further research could be done on that specific outcome to investigate more carefully. Both the salmon and alfalfa seeds experiments were promising indications of EO water's sanitation ability in food products.

Washing strawberries and other fresh produce with EO water not only can decontaminate bacteria to prevent diseases but can also increase shelf life. *Escherichia coli* K12 is a nonpathogenic strain that is widely used in commercial and research purposes. It is a good model microorganism and also one of the most extensively studied microorganisms. Therefore, its nalidixic acid and streptomycin sulfate (NSR) resistant strain is selected as the model microorganism to be studied in this project. A shelf life study is conducted to investigate the difference between treated strawberries and untreated strawberries in terms of the period of time they stay in good quality as well as the bacterial populations at both room and refrigerated temperature. In addition, quality test on color changes is also done to investigate whether EO water treatment would affect the appearance of the strawberries. It is very important, because being able to process them and allow them to stay in good condition will help with the quality of the fruits and the sale in the market. Moreover, it would also help enhance the marketability, because consumers will be more willing to purchase them if they could stay in good quality for longer period of time.

Chapter 2

Materials and Methods

Strawberries were purchased from a local grocery store in State College, PA. All strawberries were stored in the refrigerator until use. One whole strawberry was used in each trial and weighed before experiments. The average weight was in between 20 to 21 g.

Preparation of Inoculum

One strain of *E. coli* K12 resistant to nalidixic acid and streptomycin sulfate (NSR) was obtained from the Center for Food Safety, University of Georgia. The strain was grown at 37°C for 24 h in medium consisting of tryptic soy broth (TSB) supplemented with 100 μg/ml nalidixic acid and streptomycin sulfate and 0.6% of yeast extract. By adding nalidixic acid and streptomycin sulfate into the medium, the resulting selective agar could help prevent the growth of other microorganisms. The culture broth was prepared by mixing the medium with 100 ml of deionized water followed by sterilization, inoculation of 10 ml of *E. coli* K12 NSR strain medium, and incubation at 37°C for 24 h. After centrifuging the culture at 4,000 rpm and 4°C, for 10 min, the supernatant was decanted and the pellet was mixed with 50 ml of sterile 0.1% peptone water.

Inoculation of Strawberries

Surface of each strawberry was inoculated by spreading 20 μl of the prepared *E. coli* K12 NSR culture with the sterile pipettor tip. After inoculation, strawberries were kept in the laminar flow hood at room temperature (~22^{o}C) for 30 min. The resulting population on strawberries was ~7.4 $\log_{10}$ CFU per strawberry.

Preparation of electrolyzed oxidizing water

Electrolyzed oxidizing water was obtained from an EO water generator (Figure 2).

Figure 2. EO water generator (Model ROX 60 SA, Hoshizaki Electric Co. Ltd., Japan).

Salt is added into the generator chamber and low flow rate was applied. In this experiment, the acidic EO water was used for decontamination and sanitation. The acidic EO water was allowed to run from the generator for about 5 min before collecting. The pH and oxidation reduction potential (ORP) were tested by a pH and ORP meter with probes (Model 445, Corning, Inc., Big Flats, NY). Free chlorine concentration was determined by N,N-diethyl-*p*-phenylenediamine Ferrous Ethylenediammonium Sulfate (DPD-FEAS) test kit according to manufacturer's specification (Hach Co., Ames, IA). On average, the acidic EO water had a pH of 2.4, ORP of 1180 mV, and free chlorine concentration of 80 ppm.

Treatment of Inoculated Strawberries

Water baths were preset at room temperature of 22°C. A total volume of 300 ml of acidic EO water was poured in 500 ml beakers and placed into the water bath to maintain the

temperature at 22°C. Once the strawberries were ready for treatment, which was 30 min after the inoculation time, they were carried by sterilized tongs and placed into the beakers and soaked for 15 min. In order to determine the minimum and maximum parameters for time, temperature, and concentration variables, several preliminary experiments were conducted, which suggested treatment temperature from 22-35°C, time limits from 5 to 30 minutes, and concentration of acidic EO water from 50% to 100%. A Box-Bhenken response surface design is generated from MINITAB 13.30 (Minitab Inc., State College, PA.) to conduct all 15 combinations of treatment time, concentration and temperature as shown in Table 1. As a control, the untreated strawberries were inoculated with 20 µl of *E. coli* K12 NSR culture as well; however, they were then left untreated and dried in the sterile hood to be used in the comparison of untreated versus treated strawberries.

Microbiological Analysis

In order to determine the population of *E. coli* K12 NSR on treated and untreated strawberries after treatment, the strawberries were placed into sterile stomaching bags with integral filter with pre-sterilized 200 ml of 0.1% buffer peptone water in it to neutralize the effect of remaining EO water on the strawberries. Each bag was pummeled for 1 minute at 200 rpm in a stomacher (Model 400, Seward, England). The solution was serially diluted in sterile 0.1% peptone water to the 3rd dilution and spiral plated onto the Tryptic Soy Agar (TSA) with NSR agar plates by an automatic spiral plater (Autoplate 4000, Spiral Biotech, Norwood, MA). The plates were then placed into the incubator at 37°C for 24 h. After incubating, the colonies on the plates were counted using Q count system (Spiral Biotech, Norwood, MA).

Table 1. Box-Benken response surface design for EO water treatment of strawberries.

Run Order	Time (min)	Temperature (°C)	Acidic EO Water Concentration (%)
1	30	35	75
2	17.5	28.5	75
3	17.5	22	50
4	5	28.5	100
5	30	28.5	100
6	5	35	75
7	5	28.5	50
8	17.5	35	100
9	17.5	22	100
10	30	22	75
11	17.5	28.5	75
12	5	22	75
13	30	28.5	50
14	17.5	35	50
15	17.5	28.5	75

Quality Study

The color of the strawberries after soaking in EO water treatment was studied to ensure that the quality and the appearance do not have noticeable changes. Minola Chromo Meter CR200 (Minolta Inc., Ramsey, NJ) colorimeter was used to measure L*, a*, and b* color space (CIELAB). The CIELAB colors spaces were utilized by using L* to represent lightness and a* and b* to indicate chromaticity coordinates. Furthermore, the following parameters were used in analyzing the color: -a* represents green color, +a*represents red color, -b* indicates blue color, and lastly +b* indicates yellow (Bialka and Demirci, 2007). ΔE value was calculated based on all other color parameters measured to get the overall color measurement. ΔE is the "illumination distance metric of the International Commission (CIE). Three strawberries spots were chosen randomly on the untreated and treated samples, where the untreated strawberries were soaked in

DI water as a control, and the treated strawberries were treated with EO water at the optimum condition (80% EO water solution at 32°C for 30 min).

By using the following equation, ΔE = (ΔL*^2+Δa*^2+Δb*^2)^0.5 (Baur et al., 2005), the color changes between treated and untreated samples are calculated.

Shelf Study

Shelf life study was conducted to investigate the difference between treated and untreated strawberries that were placed both at room (~22^{o}C) and refrigerated temperature (~4^{o}C). Pictures were taken every day for 7 days to observe the difference in appearance of the strawberries at room temperature; the pictures of samples in the refrigerator were taken every other day for two weeks.

Chapter 3

Results and Discussion

Strawberries were inoculated with *E. coli* K12 NSR and by microbial analysis and MINITAB 13.30 (Minitab Inc., State College, PA.) software, the optimal treatment combination of temperature, time, and concentration was determined to be at 32°C by 80% EO water for 30 min.

The raw data results showed that the maximum log reduction achieved was 7.8 log with the minimum being 0.4 log reduction (Table 2). There were five experimental trials that resulted in maximum reduction of 7.8, which showed the potential ability of EO water for disinfecting and sanitization. A few trials yielded a minimum log reduction due to short treatment time, low treatment temperature or low EO water concentration. The 15 preliminary trials were used in optimization and statistical analysis to help determine the optimal condition for the treatment.

Statistical Analysis

Data were collected, in Table 2, and analyzed as the log reduction in microbial population in MINITAB 13.30 (Minitab Inc., State College, PA.) software to find the optimal conditions. Figure 3 below shows the normal probability plot, obtained from plotting the data points. It shows the data points aligned closely to the trend line, which indicates the consistency of the data and a tendency of the trend in a straight line.

Table 2. Summary of experimental data on log reduction in *E.coli* K12 NSR population.

RunOrder	Time (min)	Temperature (°C)	Acidic EO Water Concentration (%)	Log Reduction
1	30	35	75	7.8
2	17.5	28.5	75	7.8
3	17.5	22	50	1.7
4	5	28.5	100	1.4
5	30	28.5	100	7.8
6	5	35	75	2.7
7	5	28.5	50	0.4
8	17.5	35	100	1.8
9	17.5	22	100	1.9
10	30	22	75	2.1
11	17.5	28.5	75	7.8
12	5	22	75	3.9
13	30	28.5	50	3.1
14	17.5	35	50	2.7
15	17.5	28.5	75	7.8

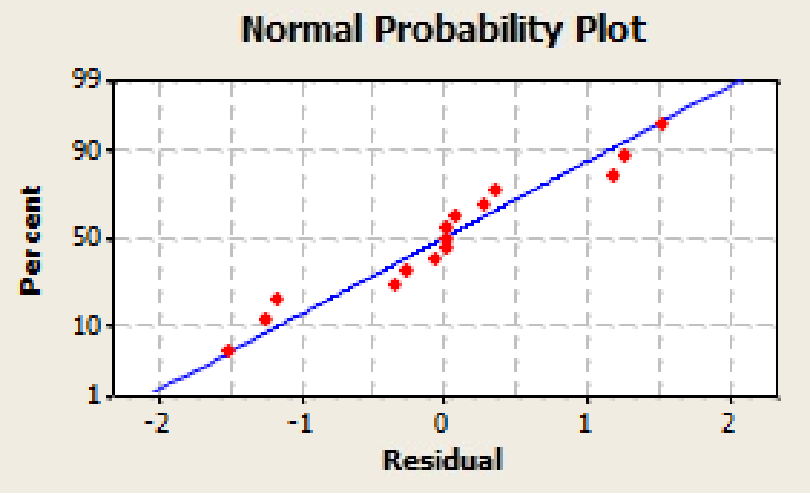

Figure 3. Normal probability plot obtained from the data.

Upon obtaining a good normal probability distribution, a set of 3D surface plots was generated based on the raw data; they are used to see the trend as well as the optimal value based on the raw data input. Figure 4 shows the surface plots, where each plot has two parameters evaluated and the lightest spot shown in each surface represents the optimal value.

Each of the 3D surface plots represented the interaction of two parameters, the curves showed the behavior of the variables. The lightest spot shown on the tip of the curve indicated the optimal value between the two parameters that were evaluated in the plot. The first plot showed the relationship between time and temperature, the second plot next to it represented the curve between time and concentration, and lastly, the surface plot on the bottom evaluated between temperature and concentration parameters.

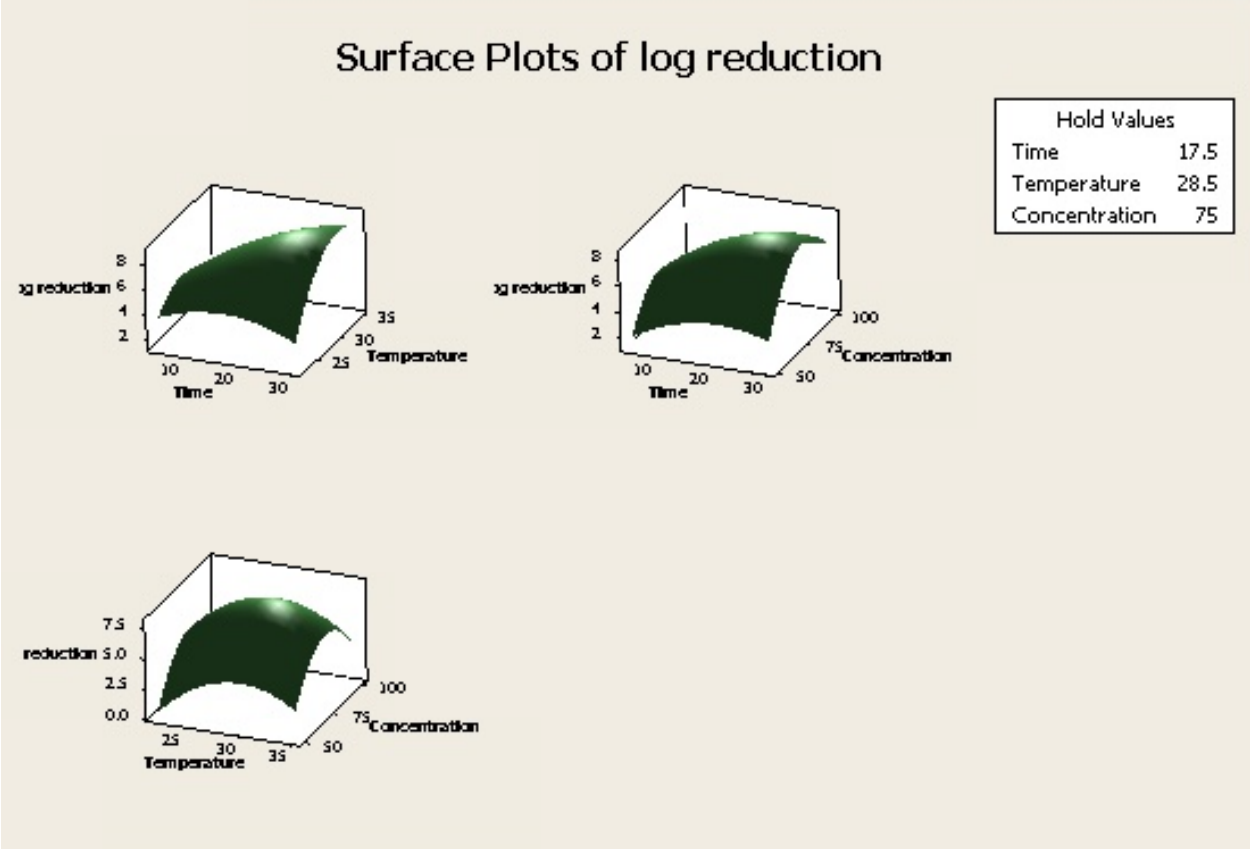

Figure 4. Surface plots of log reduction with parameter interactions.

MINTAB software also provided the optimizer option, where an exact point of optimal value for each parameter is evaluated and calculated (Figure 5). Therefore, it was clearly indicated what the values were in Figure 4 at those lightest points on the surface plots. As a result, the optimal condition was with 80% EO water for 30 min at temperature of 32°C.

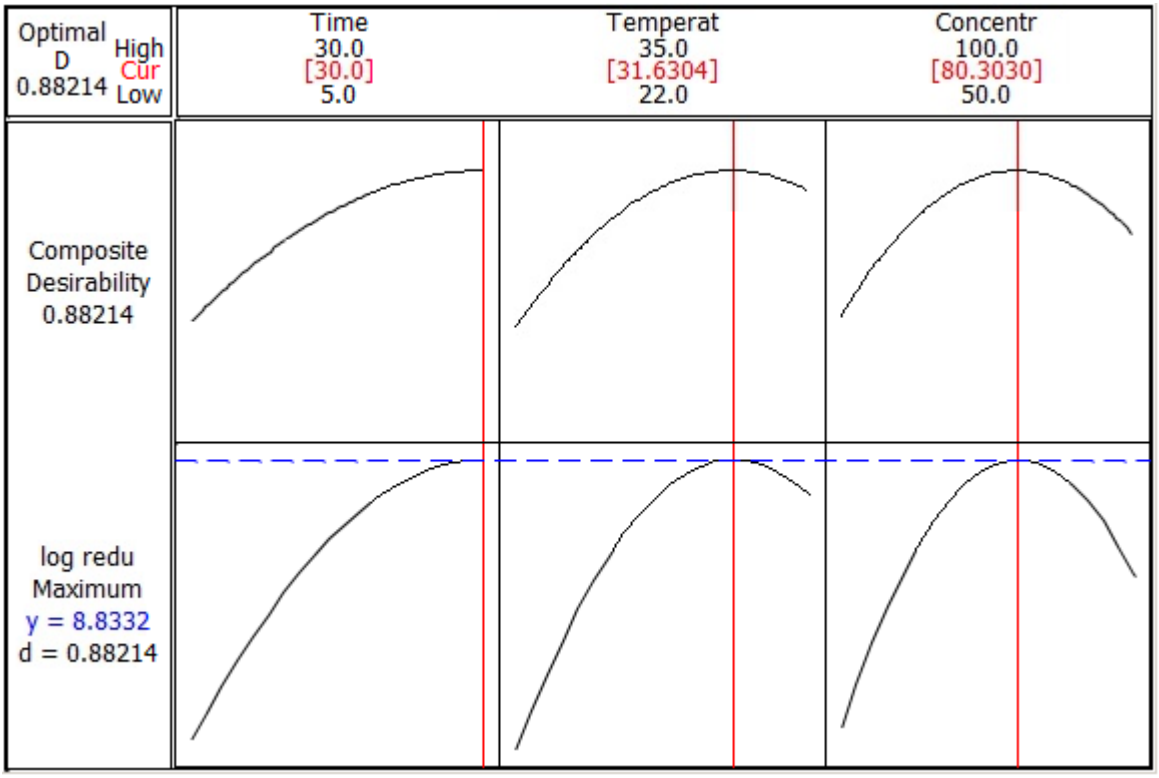

Figure 5. Optimal condition from Minitab optimizer.

From the statistical results, a regression model was acquired to predict the log reduction in microbial population given time, temperature, and acidic EO water concentration as variables.

Log reduction =-67.465-(0.4203×time)+(3.114×temperature) +(0.8284×concentration)-(0.0081×time^2)-(0.0571×temperature^2)-(0.0054×concentration^2)+(0.0212×time×temperature)+(0.003×time×concentration)-(0.0017×temperature×concentration).

Figure 6 shows the comparison between the experimental log reduction results and the estimated log reduction from the regression model. The coefficient of determination, R^2, is 0.9, which shows that the predicted model depicted the experimental data well. In addition, the slope of this line was also 0.9, which indicates a well correlation between the estimated value and experimental value. Both the R^2 and slope values are very close to 1, which is a great indication showing that estimated log reduction value calculated from the model would be close to experimental value.

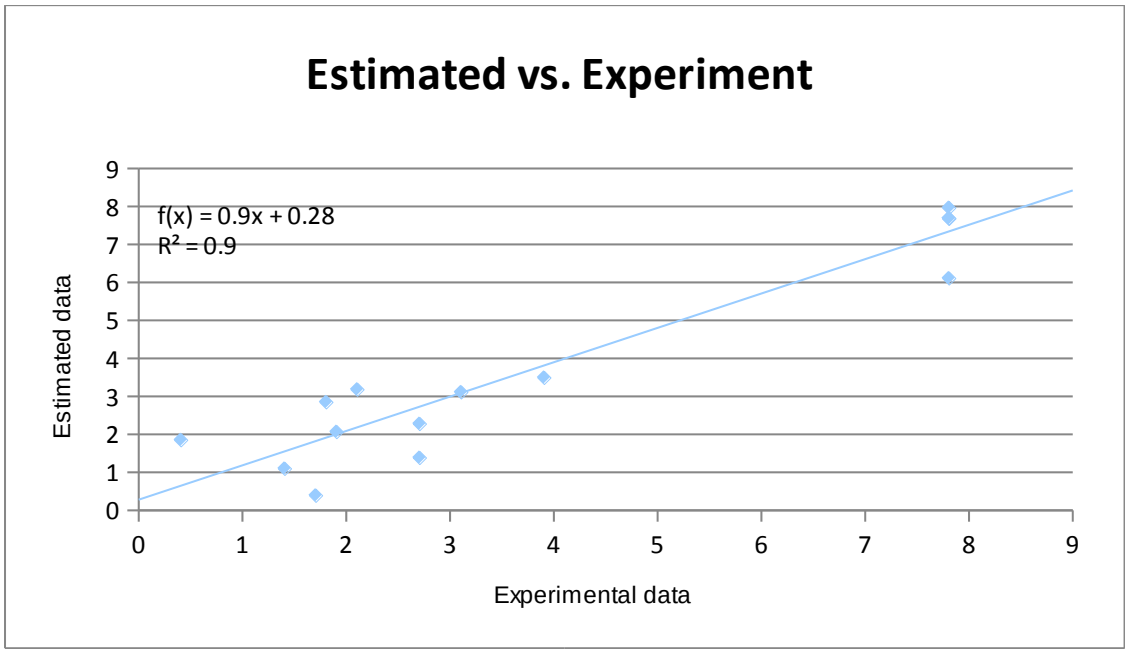

Figure 6. Experimental and estimated data correlation.

After determining the optimal condition, five replications were conducted at the determined optimum condition to validate the optimal condition. Complete inactivation was achieved in two of the five validation runs, which means no bacterial colony grew on the plates. In addition, another validation run showed almost complete inactivation, having only two CFU on

the zero dilution plate. The solution was inoculated into TSB medium with both NSR and without. After 24 hours of incubation, there was sign of bacteria growing back; therefore, the microorganism was injured, not completely dead.

Quality Study

Color tests were conducted to ensure the quality of the strawberries and the results are shown in Table 3.

Table 3. Colorimeter readings for the quality test.

Sample type	ΔL*	Δa*	Δb*	ΔE
Control	3.807±1.642A	0.240±0.431A	-0.403±2.995A	4.603±1.462A
Treated	4.223±3.584A	2.467±2.474A	2.60±5.790A	7.607±3.425A

ΔL* is the parameter in the colorimeter measured and evaluated based on the lighting of the sample, Δa* and Δb* evaluated the level of red and green color in the sample, respectively. Lastly, ΔE is the overall color measurement that took the previous color parameters into consideration to come up with an overall value that determined whether there were significant changes in the color between the untreated and treated samples. The results indicated that all values fall within the same group, which means there is no significant difference between the control and the treated strawberries. Therefore, EO water is indicated to be a good decontamination method because it does not change the appearances of the strawberries significantly.

Shelf life study

Shelf life study was conducted by naked eye observation. All the strawberry samples were picked from a box of freshly bought strawberries at the local grocery store, and they were all chosen under careful observation and screening to ensure they were in perfect quality as well as consistency before the experiment started (Figure 7). Figure 8 represents the samples placed at room temperature for one week; the top and bottom row each represents untreated and treated samples, respectively. In order to keep strawberries fresh, they are required to be stored in the refrigerator; therefore, by keeping them in room temperature, it is expected to start seeing molds growing in a couple days. During the first three days, the strawberries still looked normal as to before treatment, and there was no obvious difference found between samples. However, on the fourth day, a brown spot was observed and captured in picture on the untreated sample whereas the treated sample still looked fine. Starting on day 5, there were molds growing on both untreated and treated samples, which is inevitable, since microorganisms started to grow into lag-phase and speed up rotting process. However, the progress and the growth of molds on the strawberries were relatively slower on the treated samples. Evidently, the untreated strawberry was very moldy and extremely rotted to the point where there was liquid from the fruit in the sterile stomaching bag in the last picture. In comparison, the treated one stayed only moldy till day 7 and there was no liquid from the rotted sample.

Figure 9 shows a series of pictures of the strawberries placed in the refrigerator (4°C). The samples chosen were very fresh and in excellent quality, and since they were placed in a refrigerator, no actual mold was detected and captured throughout the 2-week period. One rotten spot was found the 15^{th} day, the last day of recording, on the untreated sample. In comparison, the treated samples remained in good quality and there were no signs of molding occurring at all.

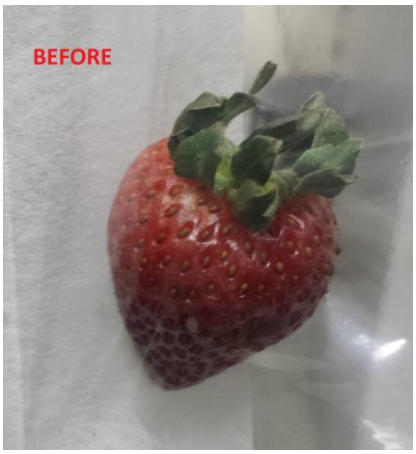

Figure 7. Fresh strawberry before treatment.

Figure 8. Series of pictures of the samples at room temperature.

Figure 9. Series of pictures of the samples at refrigerated temperature.

Chapter 4

Conclusion

The results of this study showed that EO water has potential to be used as a decontamination method on strawberries. The optimal condition to use EO water for treating strawberries is found to be at temperature of 32°C with 80% EO water for 30 min to achieve a 7 log reduction in microbial population. Five runs of validation experiments were conducted and two of them showed complete inactivation and a third one indicated almost complete inactivation, having only 2 CFU on the zero dilution plate. Enrichment test was also conducted, and there was microbial growth visibly seen in the test tubes after 24-hour incubation, which meant the bacteria was only injured and not killed from the treatment. Optimization is analyzed by MINITAB software and a regression model was generated that could be used to estimate the log reduction upon a given temperature, time and concentration condition.

Quality test was accomplished by measuring the color of the samples before and after the treatment. By two-way ANOVA statistical analysis, the results showed that there was no significant difference in terms of color changes. The results of the shelf life study indicated that the treated samples could last relatively longer without molds growing on them; in addition, the untreated samples were extremely rotten and moldy and also had liquid extracted out of the rotten fruit at the end of the week. However, the refrigerator-stored samples stayed in excellent quality after 15 days whereas the untreated sample started getting moldy on the 15^{th} day storing in the refrigerator. Overall, this project shows that EO water could potentially treat the strawberries without affecting the quality; however, further research is still needed to validate the results.

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Education

The Pennsylvania State University University Park, PA

- Bachelor of Science Biological Engineering specialized in Food and bio-processing
- Schreyer Honors College
- Member in Alpha Epsilon, an honor society for outstanding agricultural, biological and food engineers

Professional Experience

- Medicinal chemistry Intern, TEVA Pharmaceuticals, West Chester, PA
  June-August 2013
  - Carried out reactions for projects and routine laboratory work as directed by supervisor
  - Recorded results in laboratory notebook with sufficient detail and clarity
  - Responsible for performing calculations on experimental results

Honors and Awards

- David Suarez Memorial Scholarship
- Academic Excellence Scholarship
- University Scholarship in Delaware County
- Frank A. And Leona R. Taucher Scholarship
- Buckwheat Scholarship
- George Shute Scholarship in Biological Engineering
- Freshman Academic Awards
- Academic Excellence Awards

Extracurricular Leadership/Activities

- Director of Commonwealth Relations, Penn State Fresh Start Day of Service Student Organization, 2012-2013
  - A day of service organization at Penn State that is organized by directors putting together a one day of service with over a thousand students a day contributing over 2,000 hours of service to the State College community in one day
- Treasurer of Penn State Canstruction Team, 2011

    - A registered nonprofit organization that holds annual design and build competitions to construct structures made entirely out of canned food, and all the cans are donated directly to local food bank, Philabundance.
- Jared Box Project for the Ronald McDonald House, 2011
    - Fundraising, donations from people to help create activity boxes for children receiving extended medical treatment
- Planting a Seed Service Project, 2010
    - Selling apples around campus and using the money to buy gym equipment for Taggart Elementary School

Professional Presentations

- *The Transformation of My Life.* Presented at EURECA-Exhibition of Undergraduate Research and Creative Accomplishment, Penn State Brandywine, Media, PA, March 2011.
- *Factors that Affected Penn State Brandywine Students' Level of Awareness and Acceptance Pertaining to Genetically Modified Foods.* Presented at 2012 Sigma Xi Research Conference at Saint Joseph University, Philadelphia, PA, April 2012.
- *Decontamination of E.coli K12 on Strawberries*. Presented at Gamma Sigma Delta Undergraduate Research Expo, Penn State University, March 2014.

Publications and Papers

- *The Transformation of My Life*. Best of Freshman Writing by Pennsylvania State University, v.16, p. 39-40. March 2011