POTENTIAL OF ACTIVATED PERSULFATE IN FOODBORNE PATHOGEN INACTIVATION AND FRESH PRODUCE SANITATION

by

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(Under the Direction of Yen-Con Hung)

ABSTRACT

Chlorine is the most widely used sanitizing chemical in the U.S. fresh produce industry. However, chlorine can react with organic matter and produce toxic disinfection by-products, which pose potential threats to consumer health. The main objective of this project was to evaluate the efficacy of activated persulfate as an alternative sanitizing chemical for pathogen inactivation and produce sanitation. Five research chapters were conducted. The first two research chapters systematically evaluated the efficacy of ferrous and alkaline activated persulfate in inactivating *Escherichia coli* O157:H7 and *Listeria monocytogenes* in pure solution. It was found that more than 8 log CFU/mL reductions can be achieved on the two pathogens by the two activation treatments. The efficacy was dependent on the persulfate to activator ratio, persulfate concentration, and treatment time. Free radicals were determined to be the primary compounds in pathogen inactivation. The third research chapter investigated the possible pathogen inactivation mechanisms. It was found that after activated persulfate treatment, there were significant intracellular leakage of protein and DNA. The dehydrogenase activity was also inhibited. Electron microscopy images further revealed the damage on cell surfaces and cytoplasm. Therefore, it was concluded that the major inactivation mechanism was through

direct damage on cell envelope structures that caused intracellular material leakage and finally cell death. The fourth research chapter investigated the effectiveness of activated persulfate treatment for fresh romaine lettuce decontamination. It was found that up to 3.5 log CFU/g pathogen can be removed from romaine lettuce surface in 5 min without significant impact on the color quality. The efficacy was dependent on both persulfate concentration and treatment time. Furthermore, cross-contamination through wash water can be prevented at appropriate persulfate levels for both activation methods. The fifth research chapter evaluated the potential effect of organic load on activated persulfate. The overall results showed ferrous activation was easier to be affected by organic matter compared with alkaline activation. UV254 was deemed as an appropriate parameter to indicate the organic load effect. The overall findings of this project indicated activated persulfate has great potential to be applied as an alternative sanitizing chemical for fresh produce sanitation.

INDEX WORDS: Advanced oxidation process, Free radical, Foodborne pathogen, Chlorinated disinfection by-product, Alternative sanitizer

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DEDICATION

I dedicate this dissertation to my fiancée, Jia Feng, who I met before I came to UGA and stayed with me through my 6-year of UGA life; to my parents Hengjun Qi and Guoping Yang for their selfless support.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

Fresh produce contain numerous nutrients, such as vitamins and minerals, and have been considered as important components of daily diet (Meireles, Giaouris, & Simoes, 2016). As a result, the fresh produce industry has been expanding dramatically during the past few decades and has become an important part of the global food market (Joshi, Mahendran, Alagusundaram, Norton, & Tiwari, 2013). However, more and more cases of foodborne illnesses and outbreaks were found to be associated with the consumption of fresh produce (Gil, Selma, López-Gálvez, & Allende, 2009). For example, 27% of outbreaks and 40% of foodborne illnesses were linked to fresh produce consumption in 2014 (CDC, 2014). Fresh produce can provide habitat for pathogenic bacteria due to the high moisture content and natural openings on the surface (Carlin, 2007). The contamination of foodborne pathogens can take place in both pre- and post-harvest phases (Yeni, Yavas, Alpas, & Soyer, 2016). More importantly, once contaminated, the removal of pathogens will be difficult because fresh produce are minimally processed and there is no kill step for complete pathogen removal (Luo et al., 2018). Therefore, after harvest, fresh produce must be effectively washed and cleaned to minimize the potential pathogen contamination. In the U.S., chlorine-based sanitizers (e.g. sodium hypochlorite) are the most commonly used sanitizing chemical for fresh produce sanitation (Feliziani, Lichter, Smilanick, & Ippolito, 2016).

Chlorine is widely used because of the relatively low cost and high efficacy in pathogen inactivation. However, recent studies have raised concerns about using chlorine for produce sanitation. The major concern is the production of chlorinated and toxic disinfection by-products

(DBPs), such as trihalomethanes (THMs) and haloacetic acids (HAAs) (Legay, Rodriguez, Sérodes, & Levallois, 2010). These DBPs have great potentials to cause adverse effect on human health. For example, Wright, Evans, Kaufman, Rivera-Nunez, and Narotsky (2017) found high rates of birth defects were associated with pregnant women who have exposures to DBPs in drinking water. Studies have shown that chlorine washing treatments can generate DBPs residues on fresh produce (Gómez-López, Marín, Medina-Martínez, Gil, & Allende, 2013; Jose Cardador & Gallego, 2012; López-Gálvez et al., 2010). Many EU countries have banned the use of chlorine for produce wash, while no regulations have been implemented in the U.S.

Alternative sanitizing chemicals are needed to replace chlorine to reduce DBPs production and ensure fresh produce safety from microbiological hazards. Activated persulfate is a promising alternative that has shown great potential but has not been fully studied. Activated persulfate is an advanced oxidation process that relies on the production of free radicals to effectively degrade recalcitrant chemicals (Matzek & Carter, 2016). Many activators can be used for persulfate activation, such as transition metals, UV light, alkaline reagents, heat, and electrochemical power. Once activated, sulfate radicals can be produced and will further react with water or hydroxide ions to produce hydroxyl and superoxide radicals (Matzek & Carter, 2016). Activated persulfate can effectively degrade numerous chemicals, such as trichloroethylene (Liang, Lin, & Shih, 2009), perfluorooctanoic acid (Yin, Hu, Song, Liu, & Lin, 2016), and ibuprofen (Paul, Naik, Bhardwaj, & Varshney, 2014). However, its efficacy in inactivating microbes, especially foodborne pathogens, is rarely reported. To date, only one publication (except the publications by us) has reported the potential of ferrous activated persulfate in removing *Escherichia coli* O157:H7 in water solution (Wordofa, Walker, & Liu,

2017). A lot more information remains unknown and there are great research and application potentials in activated persulfate.

This dissertation systematically explored the efficacy of activated persulfate in pathogen inactivation and its application potential for fresh produce sanitation. The main hypothesis is: Activated persulfate solution is capable of inactivating and removing foodborne pathogens from fresh produce surface, and the efficacy is dependent on the activator, activator to persulfate ratios, the type of pathogen, and the organic load.

To test the main hypothesis, four research objectives were designed:

Objective 1. Systematically evaluate the efficacy of ferrous sulfate and sodium hydroxide activated persulfate in inactivating *E.* coli O157:H7 and *L. monocytogenes* in cell suspension.

Objective 2. Elucidate the inactivation mechanism of ferrous sulfate and sodium hydroxide activated persulfate on *E. coli* O157:H7 and *L. monocytogenes*.

Objective 3. Evaluate the effectiveness of ferrous sulfate and sodium hydroxide activated persulfate solution in reducing *E. coli* O157:H7 and *L. monocytogenes* from fresh produce surface in relation to the treatment conditions and compare with other chemical sanitizers.

Objective 4. Evaluate the effect of organic load in wash water on the disinfection efficacy of ferrous sulfate and sodium hydroxide activated persulfate on foodborne pathogens.

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CHAPTER 2

LITERATURE REVIEW

2.1 Fresh Produce Industry and Market

Fresh fruits and vegetables are essential components for daily diet due to the high content of nutrients, including minerals (e.g. iron, manganese, and ferrous), vitamins, polyphenolics, carotenoids, and glucosinolates (Asmita, Pragati, & Manvika, 2016; Yeni, Yavas, Alpas, & Soyer, 2016). Consumption of fresh produce can help protect consumers against eye diseases, cancers, and cardiovascular diseases (Asmita et al., 2016). Because of the numerous benefits of fresh produce consumption and the increasing trend of eating healthy, the fresh produce industry has become one of the most important and ever-growing sectors of the global food market (Joshi et al., 2013). The dramatic expansion of the produce market can be demonstrated by comparing the U.S. produce market data between 1987 and 1997. For example, the total value of the fresh produce market increased from $34.6 billion in 1987 to $70.8 billion in 1997 (Kaufman, 2000). The consumption of fresh produce also increased from 284 pounds per capita in 1987 to 319 pounds per capita in 1997 (Kaufman, 2000). In addition, the imported amount of fresh produce was doubled within the decade ($2.0 billion to $4.1 billion) (Kaufman, 2000). In 21st century, there are over 345 different types of fresh produce for sale in typical grocery stores (Garrett et al., 2003). The total value of the U.S. fresh produce market has reached $104.7 billion in 2016 and is estimated to keep increasing in the next decade (Grand View Research). Therefore, it is of paramount importance to ensure the fresh produce quality and safety to accommodate with such huge industry expansion.

2.2 Fresh Produce Safety

The trend of increasing consumption of fresh produce coincided with the increasing cases of foodborne illnesses. Illnesses caused by foodborne pathogens are prevalent all over the world. A total of 19,119 outbreaks of foodborne illness were reported by CDC from 1998 to 2015 (CDC) in the U.S. Among these outbreaks, 373,531 illnesses, 14,681 hospitalizations, and 337 deaths were recorded. The economic costs were over 50 billion dollars per year with more than 48 million people affected (Joshi, Mahendran, Alagusundaram, Norton, & Tiwari, 2013). Epidemiological studies have found an important portion of recent foodborne outbreaks were associated with the consumption of fresh produce (Yeni et al., 2016). Outbreaks associated with fresh leafy green vegetables were found to be more than any other single food type and have caused a total of 606 outbreaks, 20,003 illnesses, 1030 hospitalizations, and 19 deaths from 1973 to 2012 (Herman, Hall, & Gould, 2015).

The reason why fresh produce was associated with such a high number of outbreaks is mostly because fresh produce is usually consumed raw, and the produce freshly harvested from the fields is not thermally cooked but only minimally processed to maintain freshness before being put into the market (Yeni et al., 2016). Contamination of fresh produce may happen during both preharvest phase (e.g. seeds, dust, agriculture water, insects) and postharvest phase (e.g. handling, processing, transportation, and cross-contamination through washing) (Yeni et al., 2016). If the contamination occurs in the early phase of produce production, the risks of further spreading and cross-contamination of pathogenic bacteria are very high (Gorny, 2006). Common foodborne infections will cause both mild and severe symptoms. Mild symptoms include vomiting, diarrhea, fever, abdominal pain,headaches, muscle aches, and fatigue (Yeni et al., 2016). Severe symptoms include meningitis, septicemia, bloody diarrhea, hemolytic uremic

syndrome, and even death (Yeni et al., 2016). Although foodborne pathogens do not have specific target populations, some particular groups are more sensitive, such as pregnant women, infants, elderly, and immunocompromised people (Forsythe, 2010).

When discussing fresh produce-related foodborne pathogens, two pathogenic bacteria are generally of top concern, which are pathogenic *Escherichia coli* and *Listeria monocytogenes*.

2.2.1 Pathogenic E. coli

E. coli is a gram-negative, rod-shaped, non-spore-forming, and facultative anaerobic bacterium that is part of normal gut bacterial flora in humans (Behling et al., 2010). Six pathogenic groups of *E. coli* have been identified and grouped based on their illness symptoms and pathogenesis mechanism: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAggEC), and diffusely adherent *E. coli* (DAEC) (Forsythe, 2010). EHEC group is of top concern because it can cause the most severe symptoms, such as bloody diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS) (Kaufman, 2000). Shiga-toxin producing *E. coli* (STEC), such as *E. coli* O157:H7, belongs to the EHEC group (Kaufman, 2000). Ruminants are the primary reservoir for *E. coli*. Pathogenic *E.coli* can contaminate fresh produce through water contaminated with infected animal feces, soil, processing equipment, and handlers (Forsythe, 2010). Children under the age of six and elderly are at the highest risk with possible development of chronic kidney disease and failure (Yeni et al., 2016). A brief summary of recent *E. coli* O157:H7 outbreaks on fresh produce can be found in Table 2.1. All data are from the CDC National Outbreaks Reporting System database.

Table 2.1. Fresh produce outbreaks related with *E. coli* O157:H7.

Year	State	Illnesses	Hospitalizations	Deaths	Food Vehicle
2013	Connecticut	9	8	0	Lettuce
2013	California	5	5	0	Green leaf lettuce
2013	California	8	1	0	Green beans
2014	Multistate	4	1	0	Spinach
2015	Multistate	16	10	0	Romaine lettuce
2015	Minnesota	2	1	0	Apple cider, unpasteurized
2016	Colorado	9	1	0	Tomato; Cucumber; Lettuce
2016	Kansas	56	10	0	Apple cider
2017	Multistate	68	18	0	Spring salad; Baby leaf
2017	Multistate	25	9	1	Leafy greens

2.2.2 L. monocytogenes

L. monocytogenes is a gram-positive, rod-shaped, non-spore-forming, and facultative bacterium. *L. monocytogenes* can cause severe symptoms in pregnant women, newborn infants, and people with compromised immune system (Forsythe, 2010). Symptoms of listeriosis range from muscle aches and diarrhea to miscarriage and meningitis in pregnant women (Behling et al., 2010). *L. monocytogenes* can contaminate fresh produce through many environmental sources, such as water, animal feces, sewage, and soil (Yeni et al., 2016). More importantly, *L. monocytogenes* can survive in high salt concentrations and low temperatures. Thus, the standard food processing may not be able to completely remove *L. monocytogenes* contamination (Behling et al., 2010). In addition, the mortality rate of listeriosis caused by contaminated

produce is higher than other foodborne pathogens (Yeni et al., 2016). A brief summary of recent *L. monocytogenes* outbreaks on fresh produce can be found in Table 2.2. All data are from the CDC National Outbreaks Reporting System database.

Table 2.2. Fresh produce outbreaks related with *L. monocytogenes*.

Year	State	Illnesses	Hospitalizations	Deaths	Food Vehicle
2008	Multistate	20	16	0	Sprouts
2010	Texas	10	10	5	Celery
2013	Multistate	10	9	3	Frozen vegetables
2011	Multistate	147	143	33	Cantaloupe
2014	Multistate	2	2	1	Peaches; Nectarine
2014	Multistate	35	34	7	Caramel apple
2014	Virginia	2	2	0	Sprouts
2015	Multistate	19	19	1	Lettuce, prepackaged
2017	Multistate	3	3	0	Caramel apple

2.3 Fresh Produce Cleaning and Sanitation

Since fresh produce have been posing great threats to the public health, effective cleaning and sanitizing process must be implemented by the fresh produce industry to ensure produce safety. The most common practice used by the produce industry is washing and rinsing fresh produce with aqueous solutions. Gil, Selma, López-Gálvez, and Allende (2009) illustrated an appropriate washing process for keeping a good hygiene practice in fresh produce processing. Firstly, fresh produce needs to be washed with tap water to remove dirt and debris from the

surface. Then, the produce need to be immersed into a washing tank containing sanitizing chemicals. A further rinse step is optional depending on the sanitizer. Also, it is recommended that the produce movement direction should be opposite against the water flow direction to ensure high efficacy (Gil et al., 2009). Therefore, the use of effective sanitizing chemicals is critical for ensuring fresh produce safety. Currently, in the U.S., chlorine-based sanitizers such as sodium hypochlorite are most widely used.

2.3.1 Chlorine Sanitizer – Hypochlorite

Hypochlorite refers to the chlorine (Cl_2) form when dissolved in aqueous solution. It is the most commonly used chlorine sanitizer for both produce and drinking water disinfection (Cangliang et al., 2012). The produce industry usually use sodium or calcium hypochlorite for on-site application (Feliziani, Lichter, Smilanick, & Ippolito, 2016). The most important measure to indicate the efficacy of chlorine is free chlorine, which is defined as the remaining unreacted chlorine content in the solution that is available for disinfection purpose (Feliziani et al., 2016). Generally, free chlorine of 50 – 200 ppm is used for fresh produce sanitation (Goodburn & Wallace, 2013). pH is also an important factor. The pH of chlorine solutions are usually controlled at < 8 to maintain high efficacy. The pKa of hypochlorite is approximately 7.5. At pH below the pKa value, protonated hypochlorite (HClO) will be the major chlorine form and it is the most effective form in pathogen inactivation (Feliziani et al., 2016). For example, the time required to inactivate 95% *Penicillium digitatum* using chlorine at pH 7, 9, and 10 were 13.2, 29.4, and 88.4 s (Smilanick et al., 2002). In addition, the contact time or immersion time of fresh produce inside the sanitizing solution is generally short (less than 5 min) (Meireles, Giaouris, & Simoes, 2016).

Numerous studies have been done to evaluate the efficacy of chlorine solutions to inactivate and remove foodborne pathogens from produce surfaces. Table 2.3 is a summary of recent studies that used hypochlorite sanitizers to remove *E. coli* O157:H7 and *L. monocytogenes* from fresh produce surfaces. Generally, the extension of contact time and the increase of free chlorine concentration would not lead to significantly higher pathogen reductions. For example, 200 ppm of chlorine only achieved 0.5 log reduction of *E. coli* O157:H7 from spinach (Hadjok, Mittal, & Warriner, 2008) while 100 ppm chlorine achieved 1.3 log reduction of *E. coli* O157:H7 from spinach (Daisuke Nei et al., 2009). The increase of contact time from 3 min to 30 min only achieved a 0.5 additional log reduction on spinach (Neal et al., 2012). Other leafy greens, such as romaine lettuce, also did not show much higher reductions (around 2 log reduction) (Singh, Hung, & Qi, 2018). The produce surface texture may play an important role. For example, the reductions of *E. coli* O157:H7 achieved from blueberry (smooth surface texture) is much higher (4.8 log reduction) than that on broccoli (rash surface texture) (Hung, Tilly, & Kim, 2010; Pangloli & Hung, 2013). *L. monocytogenes*, on the other hand, showed a similar reduction range (0.7 – 2.1) in 5 min on fresh produce after treatment with chlorine sanitizers (Choi, Oh, & Lee, 2008; Rahman, Ding, & Oh, 2010; Singh et al., 2018).

Table 2.3. Efficacy of sodium hypochlorite solution in removing foodborne pathogens from fresh produce.

Pathogen	Concentration	Time	Log Reduction	Produce	Reference
E. coli O157:H7	200 ppm	30 min	1.0	Spinach	(Neal et al., 2012)
E. coli O157:H7	200 ppm	3 min	0.5	Spinach	(Hadjok et al., 2008)
E. coli O157:H7	100 ppm	5 min	4.8	Blueberry	(Pangloli & Hung, 2013)
E. coli O157:H7	100 ppm	5 min	1.6	Broccoli	(Hung et al., 2010)
E. coli O157:H7	55 ppm	5 min	1.2	Strawberry	(Hung et al., 2010)
E. coli O157:H7	100 ppm	5 min	1.3	Spinach	(Nei, Choi, Bari, Kawasaki, & Kawamoto, 2009)
E. coli O157:H7	100 ppm	5 min	1.9	Spinach	(Lee & Baek, 2008)
E. coli O157:H7	100 ppm	5 min	2.1	Romaine lettuce	(Singh et al., 2018)
L. monocytogenes	100 ppm	10 min	0.7	Cabbage	(Choi et al., 2008)
L. monocytogenes	100 ppm	5 min	2.2	Spinach	(S. M. E. Rahman et al., 2010)
L. monocytogenes	100 ppm	5 min	1.7	Romaine lettuce	(Singh et al., 2018)

2.3.2 Chlorine Sanitizer – Electrolyzed Water

Electrolyzed water is a relative new sanitizer that has been extensively studied for produce sanitation. The mechanism of electrolyzed water production and its potential application in the produce industry has been fully reviewed by Huang, Hung, Hsu, Huang, and Hwang (2008) and Gil, Gomez-Lopez, Hung, and Allende (2015). Briefly, electrolyzed water is generated by electrolyzing a salt (NaCl) solution in an electrolytic chamber. The chamber is divided into two cells with a membrane. Anode and cathode are placed in each cell. By passing direct current through the electrodes, electrolytic reactions will occur. Negative ions, such as chloride ions, will be attracted to the anode and oxidized into chlorine gas. Chlorine gas will further react with water to produce hypochlorous acid and hypochlorite ions depending on the pH. As a result, water produced from the anode side contains free chlorine and has oxidizing power (ORP > 1000 mV). Therefore, it is called electrolyzed oxidizing (EO) water. On the other side, the cathode cell will reduce water molecules into hydrogen gas and release hydroxide ion. As a result, water produced from cathode side contains a high concentration of hydroxide ion (pH > 10) and exhibits reducing power (ORP –800 to –900). Therefore, it is called electrolyzed reduced (ER) water.

EO water has been widely studied for pathogen inactivation and removal from produce surfaces. The main advantage of EO water against traditional chlorine sanitizers is the non-corrosiveness to skin and the lower toxicity (Huang et al., 2008). Also, the production of EO water only requires tap water and salt solution, which poses no safety concerns to workers. However, like chlorine, EO water is corrosive to metal equipment and is easy to react with organic matter causing reduced pathogen inactivation efficacy. ER water, on the other hand, does not show any pathogen inactivation efficacy due to the lack of sanitizing chemicals.

Recent studies on the efficacy of EO water in removing foodborne pathogens from fresh produce surface are summarized in Table 2.4. Generally, EO water can achieve 2 to 3 log reductions of *E. coli* O157:H7 on romaine lettuce in 5 min. The increase of free chlorine from 50 to 155 ppm did not show significantly higher reductions. Similar to chlorine solution, the produce type is also an important factor. For example, iceberg lettuce only showed 0.68 log reductions after treatment with EO water at 50 ppm for 2 min (Keskinen, Burke, & Annous, 2009). This might be due to the high stickiness of iceberg surface. Blueberry, with a much smoother surface texture, showed greater reductions (4.44 log reduction) (Pangloli & Hung, 2013). The removal efficacy has also been evaluated on *L. monocytogenes*. Similar to *E. coli* O157:H7, less than 3 log reductions were observed on leafy green produce and cantaloupe samples after treatment with EO water for 5 min. The increase of treatment time and free chlorine concentration did not show significant effects.

Table 2.4. Efficacy of EO water in removing foodborne pathogens from fresh produce.

Pathogen	Concentration	pH	Time	Log Reduction	Produce	Reference
E. coli O157:H7	100 ppm	6.5	5 min	2.3	Romaine lettuce	(Singh et al., 2018)
E. coli O157:H7	50 ppm	6.0	5 min	3.0	Romaine lettuce	(Pang & Hung, 2016)
E. coli O157:H7	155 ppm	7.5	5 min	2.2 – 2.5	Romaine lettuce	(Afari, Hung, & King, 2015)
E. coli O157:H7	50 ppm	2.6	2 min	0.68	Iceberg lettuce	(Keskinen et al., 2009)
E. coli O157:H7	30 ppm	2.l	5 min	4.44	Blueberry	(Pangloli & Hung, 2013)
E. coli O157:H7	50 ppm	2.54	5 min	1.58	Strawberry	(Hung et al., 2010)
L. monocytogenes	100 ppm	6.5	5 min	2.0	Romaine lettuce	(Singh et al., 2018)
L. monocytogenes	100 ppm	6.5	5 min	2.1	Cantaloupe	(Singh et al., 2018)
L. monocytogenes	50 ppm	6.5	1 min	2.0	Leafy greens	(Stopforth, Mai, Kottapalli, & Samadpour, 2008)
L. monocytogenes	50 ppm	2.54	3 min	2.8	Spinach	(Rahman et al., 2010)

2.4 Disinfection by-products

Although chlorine-based sanitizers have numerous advantages and benefits for fresh produce sanitation, concerns have been on the rise regarding the generation of chlorinated and toxic disinfection by-products (DBPs). Sodium hypochlorite, due to its strong oxidizing power, can react with organic matter (from fresh produce during washing processing) to generate toxic DBPs, such as trihalomethanes (THMs) and haloacetic acids (HAAs) (Richardson, 2003). Major THMs of concerns are chloroform, bromoform, bromodichloromethane, and dibromochloromethane. Major HAAs of concerns are chloroacetic acid, dichloroacetic acid, trichloroacetic acid, bromoacetic acid, and dibromoacetic acid (Sérodes, Rodriguez, Li, & Bouchard, 2003). Among them, chloroform and bromodichloromethane have been classified as possible carcinogens to humans by WHO's International Agency for Research on Cancer (Gil et al., 2015). There are also other types of DBPs that are potential concerns, such as haloacetonitriles, haloketones, haloaldehydes, and chloropicrin (Sérodes et al., 2003).

Both chronic and acute health effects can be caused by DBPs consumptions. Wright, Evans, Kaufman, Rivera-Nunez, and Narotsky (2017) found pregnant women who were exposed to drinking water with DBPs have a much higher risk of delivering babies with cardiovascular defects compared to women who have no exposure to DBPs. A strong correlation was also found between DBPs exposure and colon or bladder cancer by epidemiologists (Cristina et al., 2004; Rahman, Driscoll, Cowie, & Armstrong, 2010). THMs were deemed as the primary DBPs in causing colon cancers. For example, Costet et al. (2011) found an odds ratio of 1.27 for the relation between average THMs ingestion and bladder cancer risk in men. Odds ratio larger than 1 indicated a positive association between THMs ingestion and bladder cancer. An odds ratio of 1.61 was also found between THMs consumption and bladder cancer (Kenneth et al., 1998).

Tumors could also be caused when HAAs exposure was established. For example, liver tumors were found in rats fed with long-term HAAs (Florentin, Hautemanière, & Hartemann, 2011). In addition to cancer and tumor risks, other health symptoms may also be caused by DBPs exposure and consumption. Studies have suggested that the DBPs in swimming pools could cause permanent damage to lungs and increase the risk of asthma, bronchial hyperactivity, and airway inflammation (Florentin et al., 2011).

Considering the potential toxicity of DBPs, water chlorination is tightly regulated in the U.S. regarding to DBPs residue levels in drinking water. The maximum limit for THMs is 80 µg/L and the limit for HAAs is 60 µg/L in the U.S. The use of chlorine wash for produce sanitation has been banned by many EU countries. However, in the U.S., chlorine are still approved for fresh produce sanitation. Studies have demonstrated that chlorine wash can generate DBPs residues in both wash water and fresh produce. Fan and Sokorai (2015) found that fresh-cut lettuce washed with 100 mg/L chlorine contained 14 to 22 µg/kg trichloromethane. The DBPs level can be increased up to 40 µg/kg after the lettuce samples were further processed into lettuce juice. Haute, Sampers, Holvoet, and Uyttendaele (2013) also found the total trihalomethane level in wash water reached 124.5 µg/L at COD level of 1000 mg/L. The DBPs residue level can be much higher if excess amount of NaCl are present in the wash water. Gómez-López, Marín, Medina-Martínez, Gil, and Allende (2013) demonstrated the total THMs residue level on baby spinach was about 73.4 µg/kg after 1 min immersion in EO water containing NaCl, which was much higher than the spinach samples treated with EO water or NaCl solution alone. On the other hand, HAAs were also found in over 150 beverage products, which were deemed as the results of chlorinated water usage (Cardador & Gallego, 2015). In addition, these HAAs could remain constant for more than 3 months.

From the discussion above, it can be concluded chlorinated DBPs 1) are potential toxic chemicals; 2) have been found in many fresh produce types and processed food products; 3) have potential adverse health effects. Therefore, it is of paramount importance to develop a new sanitizer that can achieve equal or higher pathogen reduction efficacy on fresh produce but generate less or no chlorinated DBPs residues.

2.5 Activated persulfate

Our research is proposing a new sanitizing chemical, activated persulfate, to be used as an alternative sanitizer for fresh produce wash. Free radicals are the main sanitizing compounds involved which are different from traditional sanitizing chemicals. Also, since no chlorine is involved, chlorinated DBPs can be reduced or eliminated.

Activated persulfate, a relatively new advanced oxidation process (AOP) that has been studied extensively by environmental scientists, shows great promise to be used as an alternative sanitizer for fresh produce sanitation. Persulfate ($S_2O_8^{2-}$), also known as peroxydisulfate, has been studied for its efficacy in degrading chemical pollutants in wastewater and soil samples (Matzek & Carter, 2016). Persulfate usually exists in the form of salts, with sodium, potassium or ammonium (Tsitonaki et al., 2010). Sodium persulfate is commonly used due to the high water solubility and benign residual products (Tsitonaki et al., 2010). Sodium persulfate is a white solid with high stability and long shelf life. When dissolved in water, persulfate ions will be formed, which is a strong oxidant (E^o = 2.1 V). Despite persulfate being a thermodynamically strong oxidant, it is not effective in degrading organic contaminants by direct reaction (Devi, Das, & Dalai, 2016). Activators, such as transition metals, UV light, alkaline pH, heat, ultra-sonication, and activated-carbon, are needed to activate persulfate to produce highly reactive

sulfate radicals (equation 2.1) for chemical degradation purposes (Matzek & Carter, 2016). Field application of activated persulfate has been conducted for contaminated soil and groundwater remediation. Tsitonaki et al. (2010) summarized the field applications from 2000 – 2007. Most of the applications targeted on removal of chlorinated ethenes, BTEX, and PAHs and the most widely used activators were ferrous.

The efficacy of activated persulfate is mainly dependent on the free radicals, such as sulfate and hydroxyl radicals. The sulfate radical has a high oxidation potential (E^o = 2.6 V) and is nonselective in chemical degradation reactions (Hao, Guo, Wang, Leng, & Li, 2014). The primary mechanisms of sulfate radicals in degrading organic chemicals are hydrogen abstraction, double bond addition, and electron transfer (Petri et al., 2011). Chemicals with electron donating groups will react quickly with sulfate radicals due to its electrophilic property (Tsitonaki et al., 2010). Sulfate radicals can also react with water to form hydroxyl radicals (Equation 2.3), which are also oxidative and effective in chemical degradation. Both sulfate and hydroxyl radicals can contribute to chain reactions and further produce more reactive intermediates that can be involved in subsequent chemical degradations (Petri et al., 2011).

$$S_2O_8^{2-} + \text{activator} \rightarrow SO_4^{-}\cdot + (SO_4^{-}\cdot \text{ or } SO_4^{2-}) \quad (2.1)$$

2.5.1 Iron Activated Persulfate

Metal elements, such as silver, copper, iron, zinc, and manganese, can be used for persulfate activation (Matzek & Carter, 2016). Among all those metal elements, iron is the most widely studied and applied as an effective persulfate activator because of the high activation effectiveness, no/low toxicity, low impact to the environment, and high cost-effectiveness

(Rastogi, Al-Abed, & Dionysiou, 2009). The general activation mechanism using ferrous ion is shown in Equation 2.2, where sulfate radical is the primary activation product. In addition, Fe^0 can also be used as an indirect activator. Fe^0 can be oxidized into Fe^{2+} by persulfate, after which it will go through the activation process as shown in Equation 2.2 (Oh, Kim, Park, Park, & Yoon, 2009). Ferrous ion concentration plays an important role in the overall persulfate activation process. If ferrous ion is insufficient, ineffective persulfate usage will be caused and less sulfate radical will be produced. If ferrous ion is in excess amount, the persulfate activation will be suppressed through the sulfate radical scavenging effect (Equation 2.4). As a result, less sulfate radical will be produced.

Numerous studies have demonstrated the high efficacy of iron activated persulfate in degrading recalcitrant chemicals, such as p-nitrophenol (Zhang, Chen, Zhou, Murugananthan, & Zhang, 2015), diuron (Romero, Santos, Vicente, & González, 2010), BDE209 (Peng, Xu, Zhang, Liu, Liu, et al., 2017), atrazine (Bu, Shi, & Zhou, 2016), propachlor (Liu, Shih, Sun, & Wang, 2012), trichloroethylene (Liang, Lee, Hsu, Liang, & Lin, 2008), BTEX (Liang, Huang, & Chen, 2008), and benzoic acid (Zhou et al., 2013). Some studies argued a minimum persulfate to iron ratio of 1:1 is needed to maximize chemical degradation efficacy (Al-Shamsi & Thomson, 2013; Zhang et al., 2015) while some studies demonstrated persulfate to iron molar ratio of 1:1.25 is more appropriate (Liang, Bruell, Marley, & Sperry, 2004). Matzek and Carter (2016) suggested the optimum ratio may vary depending on the iron form, ferrous addition methods, and target chemical.

In addition, the process of iron activated persulfate is very fast which will result in fast consumption of persulfate or iron ion and lead to limited effective time period (Liang et al., 2004). Currently, the most common way to extend the iron activation period and improve the

overall chemical degradation efficacy are sequential/continuous addition of iron and use of chelating agent (Matzek & Carter, 2016). For example, sequential addition of ferrous ion increased the degradation efficacy of bisphenol A from 49% to 97%, and continuous addition of ferrous ion lead to complete degradation of bisphenol A (Jiang, Wu, Wang, Li, & Dong, 2013). Another way to achieve continuous addition of ferrous ion is by using Fe^0 since Fe^0 will first react with persulfate to produce Fe^{2+} (Matzek & Carter, 2016). On the other hand, use of a chelating reagent for iron activated persulfate has also been explored. The primary mechanism is by trapping ferrous ion with chelating agent to achieve slow release (Matzek & Carter, 2016). For example, the degradation of aniline was improved from 45% to 60% by using citric acid as the chelating reagent (Zhang, Xie, Huang, & Liang, 2014).

$$S_2O_8^{2-} + Fe^{2+} \rightarrow SO_4^{-}\cdot + SO_4^{2-} + Fe^{3+} \quad (2.2)$$

$$SO_4^{-}\cdot + H_2O \rightarrow SO_4^{2-} + HO\cdot + H^{+} \quad (2.3)$$

$$SO_4^{-}\cdot + Fe^{2+} \rightarrow Fe^{3+} + SO_4^{2-} \quad (2.4)$$

2.5.2 Alkaline Activated Persulfate

Alkaline reagents, such as sodium hydroxide, is another commonly used activator for persulfate activation. Furman, Teel, and Watts (2010) described a detailed mechanism of activation. At alkaline conditions (pH > 10), persulfate will react with hydroxide ions to form peroxomonosulfate and sulfate ions. The formed peroxomonsulfate is extremely unstable and will quickly decompose to hydroperoxide. Then, hydroperoxide will keep reacting with persulfate ions, reducing it to sulfate radical and sulfate ions while hydroperoxide itself gets oxidized into superoxide radical. The overall process can be summarized in Equation 2.5. Under

alkaline conditions, sulfate radicals can further react with hydroxide and produce hydroxyl radicals (Equation 2.6). Therefore, in alkaline activated persulfate, three types of free radicals are present, being sulfate radical, hydroxyl radical, and superoxide radical. Higher alkaline level can increase the overall free radical levels (Furman et al., 2010). However, a persulfate to alkaline molar ratio of 1:2 is recommended (Matzek & Carter, 2016). This is because protons are continuously produced from the activation process (Equation 2.5) so that the pH will be gradually reduced to near neutral level.

$$2S_2O_8^{2-} + 2H_2O \xrightarrow{OH^-} 3SO_4^{2-} + SO_4^{-}\cdot + O_2^{-}\cdot + 4H^+ \quad (2.5)$$

$$SO_4^{-}\cdot + OH^- \rightarrow SO_4^{2-} + OH\cdot \quad (2.6)$$

Alkaline activated persulfate has been demonstrated as an effective reagent for organic chemical degradation. Peng, Xu, Zhang, Liu, Lu, et al. (2017) studied NaOH activated persulfate in degrading decabromodiphenyl ether (BDE209) in slurry soil samples. By controlling the pH at 12.2 and temperature at 25 ^oC, up to 25% BDE209 degradation was observed in 6 h and it was demonstrated that higher initial persulfate levels can achieve higher BDE209 degradation. Epold and Dulova (2015) also used NaOH as the persulfate activator. By controlling the pH at 11, they were able to achieve 25% removal of levofloxacin in 3 h. Similarly, by increasing the persulfate to levofloxacin molar ratio, higher removal efficacy was observed. Similar results were also achieved by Liang and Guo (2012) who used lime and sodium hydroxide activated persulfate to remediate diesel-contaminated soil samples. A maximum removal rate of 30% was observed. They also indicated higher alkaline levels would accelerate the decomposition process of persulfate but not necessarily lead to more chemical reductions.

2.5.3 UV light Activated Persulfate

UV light can activate persulfate. The energy contained in UV light is able to break the O-O bond within the persulfate molecule and produce sulfate radicals (Equation 2.7). UV light is more cost-effective and environmentally friendly since no other activator chemicals are included (Matzek & Carter, 2016). The two important factors in UV light activated persulfate are wavelength and UV fluence rate (Matzek & Carter, 2016). UV light at 254 nm and 365 nm are the two common wavelengths that have been studied. Lin, Lee, and Hsu (2013) achieved a 97% removal rate of polyvinyl alcohol using UV365 activated persulfate in 10 min. However, UV254 activated persulfate achieved 100% removal rate of polyvinyl alcohol in less than 5 min (Lin & Wu, 2014). It was therefore suggested UV254 is more efficient in persulfate activation which might be due to the higher energy. Fluence rate can also contribute to the degradation rate. For example, UV light activated persulfate at 30 W achieved higher and faster removal of acid blue113 (Shu, Chang, & Huang, 2015).

$$S_2O_8^{2-} + UV \rightarrow 2SO_4^{-}\cdot \quad (2.7)$$

Many other studies have also demonstrated the efficacy of UV light activated persulfate in chemical degradation. Ismail, Ferronato, Fine, Jaber, and Chovelon (2017) achieved complete degradation of sulfaclozine in 40 min with UV365 activated potassium persulfate. Sulfate radical and hydroxyl radical were found to equally contribute to the sulfaclozine degradation. Xie et al. (2015) studied the efficacy of UV254 activated persulfate in degrading 2-methylisoborneol and geosmin, and they achieved over 80% degradation rates for both chemicals in 7 min. The contribution of hydroxyl radicals in chemical degradation was estimated to be at least 2 times

higher than sulfate radicals. Furthermore, Liu, He, Fu, and Dionysiou (2016) demonstrated 85% removal rate of oxytetracycline in solution with UV254 activated persulfate in about 15 min. However, they argued the major contributing radical is sulfate radical rather than hydroxyl radical based on the analysis of degradation by-product. However, UV light activated persulfate may be limited for real application due to its poor penetration ability in water (Matzek & Carter, 2016).

2.5.4 Other Activation Methods

Other technologies were also proposed for persulfate activation, such as heat (Huang, Zhao, Hoag, Dahmani, & Block, 2005; Ji, Dong, Kong, Lu, & Zhou, 2015; Zhao, Hou, Fujii, Hosomi, & Li, 2014), hydrogen peroxide (Crimi & Taylor, 2007), electrochemical power (Govindan, Raja, Noel, & James, 2014; Songhu, Peng, & Alshawabkeh, 2014), and ultra-sonication (Hao et al., 2014; Wang et al., 2014; Zou, Zhou, Mao, & Wu, 2014). However, they are not as popular as ferrous, alkaline, and UV light because of the potential limitations. For example, heat activation is easy to conduct but heating requires particular instrument and controls on emissions (Tsitonaki et al., 2010). The cost of heating can also be much higher than ferrous and UV light. More importantly, for potential fresh produce processing, heating cannot be applied since it will destroy the freshness of produce. Peroxide, on the other hand, is cheap and easy to apply. However, peroxide is very unstable and can pose potential safety concerns (Tsitonaki et al., 2010). Electrochemical activation is environmental friendly since it can provide power for continuous regeneration of ferrous ion. However, it is limited for large scale application and can pose potential threats to workers in fresh produce processing facilities.

2.5.5 Activated Persulfate in Microbial Inactivation

The efficacy of activated persulfate in degrading chemical pollutants has been extensively studied, but its potential and effectiveness in microbial inactivation, especially for foodborne pathogens, has not been thoroughly investigated. Among the few studies investigating the efficacy of activated persulfate in microbial inactivation, the majority of the studies were targeted for environmental water disinfection where low concentration of persulfate and long treatment time were used. Persulfate activated by UVC light for wastewater treatment is most commonly studied, including *E. coli* inactivation in wastewater (Michael-Kordatou et al., 2015; Sun, Corey, & Huang, 2016), *Microcystis aeruginosa* removal in source water (Wang, Chen, Xie, Shang, & Ma, 2016), *E. faecalis* inactivation in distilled water (Moreno-Andrés, Rios Quintero, Acevedo-Merino, & Nebot, 2018), and inactivation of *Bacillus subtilis* spores (Sabeti et al., 2017). Generally, *E. coli* are easier to inactivate (over 6 log reduction in 30 min) compared with *Microcystis aeruginosa* (about 2 log reduction in 2 h) and *B. subtilis* spores (about 2.2 log reduction in 1 h) (Sabeti et al., 2017; Sun et al., 2016).

Other activation methods were also studied for microbial inactivation. For example, Samyoung, Peterson, Righter, Miles, and Tratnyek (2013) used iron (Fe^0) activated persulfate to inactivate *Pseudonitzshia delicatissima* and *Dunaliella tertiolecta* in ballast water. They achieved complete inactivation of these two microbes in 7 days at 4 mM persulfate. Heat was also utilized as an activator to inactivate *Pseudomonas putida* KT2440 (Tsitonaki, Smets, & Bjerg, 2008). However, they concluded the effect of heat activation was not as effective as the efficacy of Fenton reagent. In addition, combination of two activators were evaluated for potential enhancement of microbial inactivation. Garkusheva et al. (2017) investigated the effect of persulfate activated by UV (A+B) light with or without ferrous ion in inactivating *E. coli* in

pure water, lake water and wastewater. They found the combination of UV light and ferrous ion achieved significantly more *E. coli* reductions in all three water samples than any single activators.

On the other hand, the study of activated persulfate in foodborne pathogen inactivation is very rare. To my best knowledge, there is only one published paper that described the potential of iron activated persulfate in inactivation of *E. coli* O157:H7 (Wordofa, Walker, & Liu, 2017). No study has reported the potential of activated persulfate treatment for fresh produce sanitation. There is a big information gap and research need to fully evaluate the potential of activated persulfate for pathogenic bacteria inactivation and potential application for fresh produce sanitation.

2.5.6 Possible Mechanism of Microbial Inactivation by Free Radicals

Possible mechanisms of pathogen inactivation by free radicals have been proposed. Wang, Huang, Yu, and Wong (2015) indicated the denaturation of catalase and superoxide dismutase was the primary reason for microbial death since these enzymes can protect bacterial cells from oxidative stress. Attenuation of free radicals into non-oxidative compounds are the main function of these two enzymes (Leung, Chan, Hu, Yu, & Wong, 2008). During free radical attack, these two enzymes can be fully consumed and therefore render microbial cells to limited protection (Chen et al., 2011). As a result, proteins can be fragmented and cell viability will be lost (Bosshard et al., 2010). On the other hand, the inactivation can also be initiated from loss of integrity of cell envelope structures, including cell membranes and cell walls (Wang et al., 2015). For example, the study of Kim et al. (2013) revealed that UV light activated TiO_2 caused significant damage on the cell surface of *E. coli*, *L. monocytogenes*, *S.* Typhimurium under the observation of scanning electron photomicrograph. Damage of plasmid DNA were also

observed. Such damage on cell envelope structures can cause subsequent leakage of intracellular materials and finally result in cell death. Therefore, measurement of intracellular materials, such as K^+, was often used to evaluate the inactivation mechanism (Wang et al., 2015). The free radicals' ability in causing cell envelope structure damage and subsequent DNA damage was also demonstrated by Tian et al. (2018), Bogdan, Zarzyńska, and Pławińska-Czarnak (2015), and Zhang, Zou, Cai, Li, and He (2018).

2.5.7 Safety and toxicity of persulfate

Ammonium, potassium, and sodium persulfate are the most three common salt forms of persulfate. Sodium persulfate is the most commonly used salt for activated persulfate due to the high water solubility and benign activation product (Matzek & Carter, 2016). The safety and detailed information of sodium persulfate has been thoroughly reviewed by (Pang & Fiume, 2001). Briefly, $Na_2S_2O_8$ is the chemical formula of sodium persulfate (molecular weight 238.13 g/mol). It exists in white crystalline powder and can go through slow decomposition in water solution. Sodium persulfate is widely used in hair bleachers. Sodium persulfate has been approved as the component (< 1%) for making paper and paperboard that will have direct contact with food. The potential toxicity of sodium persulfate was not included in their review but the potential toxicity of ammonium persulfate was introduced. Ammonium persulfate was negative in Ames test, tumor promotion, carcinogenicity test, and chromosomal aberration test. Sodium persulfate was found not to be a skin sensitizer and would not cause urticarial reaction at 17.5% concentration. However, persulfate can cause skin symptoms, including irritant dermatitis, allergic eczematous dermatitis, localized contact urticarial, asthma, rhinitis, and syncope. In addition, none of the persulfate salts have been approved as GRAS (generally recognize as safe) or food additive (both direct and indirect) by U.S. FDA yet.

2.5.8 Information Needed to Adopt Activated Persulfate for Produce Washing

In order to adopt activated persulfate for produce washing, the first thing to address and evaluate is how effective can activated persulfate be in inactivating foodborne pathogens. Traditional sanitizers, such as chlorine, are able to achieve high inactivation efficacy on a wide range of microbes. For example, EO water with 5 mg/L free chlorine can achieve near 8 log CFU/mL reductions of *E. coli* O157:H7 and *L. monocytogenes* in pure solution in 30 s (Park, Hung, & Chung, 2004). The first step of evaluating the potential of activated persulfate in produce washing will therefore be studying its efficacy in inactivating foodborne pathogens in pure solution. In order to test its efficacy spectrum, both gram negative and gram positive foodborne pathogens should be tested. Potential influential factors also need to be considered. For example, the effect of initial persulfate concentration and the persulfate to activator ratio might play an important role, which is the case for chemical degradation as discussed in previous sections. Validation studies are then needed to verify if activated persulfate can be as effective as chlorine sanitizers for removing foodborne pathogens from fresh produce during washing processes. More importantly, some activators, such as ferrous and alkaline, may be able to cause detrimental effects on produce quality. Therefore, the evaluation of produce quality after activated persulfate treatment is also needed. Furthermore, free radicals produced by activated persulfate are very reactive and can react with organic matter (Chen et al., 2018; Eduard, Bertram, Claudia, Philipp, & Ralf, 2018; Yemmireddy & Hung, 2015). The efficacy of activated persulfate may be reduced in real application when the organic load in wash water builds up. Thus, the effect of organic load on activated persulfate also need to be studied.

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CHAPTER 3

EFFICACY OF ACTIVATED PERSULFATE IN INACTIVATING *ESCHERICHIA COLI* O157:H7 AND *LISTERIA MONOCYTOGENES*

Qi, H., Huang, Q., & Hung, Y.-C. (2018). *International Journal of Food Microbiology*, *284*, 40-47. Reprinted here with permission of the publisher.

Abstract

Concerns have been on the rise regarding the use of chlorine-based sanitizers for fresh produce sanitation due to the production of toxic disinfection by-products (DBPs). This study was undertaken to evaluate the efficacy of activated persulfate in inactivating *Escherichia coli* O157:H7 and *Listeria monocytogenes* in pure culture. The objectives were to study the effect of persulfate to activator ratios and determine the major contributing radical in pathogen inactivation. A five-strain cocktail of each pathogen was treated with sodium persulfate activated by ferrous sulfate or sodium hydroxide for 60 s or 120 s. Non-selective agars supplemented with sodium pyruvate were used for pathogen enumeration. The steady-state concentrations of free radicals were quantified using HPLC-DAD. Radical scavengers (tert-butanol, isopropanol, and benzoquinone) were used to determine the major contributing radical in pathogen inactivation. The results showed more than 7 log CFU/mL reductions can be achieved in 120 s for both pathogens at appropriate activation conditions. For ferrous activation, the persulfate to ferrous ratio played an important role in the overall inactivation efficacy. The maximum pathogen reduction (7.77 log CFU/mL for *E. coli* O157:H7 and 7.25 log CFU/mL for *L. monocytogenes*) was achieved at persulfate to ferrous molar ratio of 1:0.33 when the initial persulfate concentration was set at 40 mmol/L. Further increase or decrease of ferrous ratio always lead to lower pathogen reductions. For alkaline activation, the inactivation efficacy increased with more initial sodium hydroxide. The maximum reduction was achieved at 40 mmol/L persulfate with 30 mmol/L sodium hydroxide for *E. coli* O157:H7 (6.21 log CFU/mL reduction) and at 500 mmol/L persulfate with 350 mmol/L sodium hydroxide for *L. monocytogenes* (8.64 log CFU/mL reduction). Also, persulfate activated by sodium hydroxide always achieved significantly ($P < 0.05$) higher microbial reductions than sodium hydroxide or persulfate alone. *L. monocytogenes*

was generally more resistant against the activated persulfate treatment compared with *E. coli* O157:H7, which might be due to the different cell envelope structures between Gram-positive and Gram-negative bacteria. Hydroxyl radical was demonstrated to be the major radical to inactivate both pathogens in ferrous activation while superoxide radical was demonstrated to be the major radical to inactivate both pathogens in alkaline activation.

Keywords

Advanced oxidation process; Hydroxyl radicals; Superoxide radicals; Microbial inactivation; Foodborne pathogens

3.1 Introduction

Fresh fruits and vegetables are essential sources of numerous nutrients, such as vitamins and minerals (Asmita et al., 2016). The fresh produce industry has become one of the most important parts of the global food market (Joshi et al., 2013). However, the trend of increasing consumption of fresh produce coincided with the increasing cases of foodborne illnesses and outbreaks (Afari et al., 2015). This is mainly because fresh produce are suitable habitats for pathogenic microorganisms due to the high moisture and natural openings (Carlin, 2007) as well as the fact that fresh produce are usually consumed raw (Yeni et al., 2016). Chlorine based-sanitizers, such as sodium hypochlorite, are the most commonly used disinfectant chemicals in the fresh produce industry because of the low cost and high efficacy in pathogen inactivation (Feliziani et al., 2016). However, the production of toxic disinfection by-products (DBPs), such as trihalomethanes, haloacetic acids, and nitrogenous DBPs, during chlorine sanitation has drawn attention as these DBPs pose potential threats to consumers' health (Joshi et al., 2013; Legay et al., 2010). For example, one epidemiological study has suggested pregnant women exposed to DBPs have an elevated risk of delivering babies with cardiovascular defects (Wright et al., 2017). A recent study also found sodium hypochlorite washed lettuce contained significantly more toxic DBPs than unwashed controls (Lee and Huang, 2017). Alternative sanitizers that are effective in pathogen removal with less or no toxic DBPs production are therefore needed to ensure produce safety.

There have been studies that investigated alternative and novel disinfectants for produce disinfection, such as ozonated water, chlorine dioxide, and electrolyzed oxidizing water. However, all these techniques have their limitations making them unsuitable for widespread application. Ozonated water has been approved as GRAS by FDA as an effective disinfectant

against bacteria, fungi, protozoa, and microbial spores (Joshi et al., 2013). However, ozone is very unstable and can be toxic in gaseous phase by causing irritations to eyes and the respiratory system (Artes et al., 2009). Chlorine dioxide has also been considered as an alternative due to the high efficacy in pathogen inactivation and low reactivity with organic matter (Joshi et al., 2013), but the explosive potential of chlorine dioxide is a safety concern. Electrolyzed oxidizing water has been extensively studied and reported by numerous scientific publications (Gil et al., 2009; Huang et al., 2008). Although electrolyzed oxidizing water is considered advantageous compared to bleach solutions, the major active compounds are free chlorine that would still present toxic DBPs problems.

Activated persulfate, a relatively new advanced oxidation process (AOP) that has been extensively studied by environmental scientists, shows great promise to be used for fresh produce sanitation. Persulfate usually exists in the form of salts, such as sodium persulfate (Tsitonaki et al., 2010). Persulfate is a thermodynamically strong oxidant but not reactive in chemical degradation through direct reaction (Devi et al., 2016). However, persulfate can be activated by activators, such as transition metals, alkaline reagents, and UV light, to produce reactive free radicals (Eq. 3.1) (Matzek and Carter, 2016). These intermediate free radicals are highly oxidative and can degrade many recalcitrant organic chemicals, such as perfluorooctanoic acid (Yin et al., 2016), benzotriazole (Xiong et al., 2014), trichloroethylene (Liang et al., 2009), and ibuprofen (Paul et al., 2014). Although the efficacy of activated persulfate in degrading organic chemicals has been well studied, its effect on pathogen inactivation has not been fully explored. To our best knowledge, there are only two published papers that have studied the efficacy of activated persulfate in pathogen inactivation. Sun et al. (2016) found UV-254nm

activated persulfate can effectively inactivate pathogenic *Escherichia coli* while Wordofa et al. (2017) revealed ferrous activated persulfate could reduce the viability of *E. coli* O157:H7.

$$S_2O_8^{2-} + \text{activator} \rightarrow SO_4^{-}\cdot + (SO_4^{-}\cdot \text{ or } SO_4^{2-}) \quad (3.1)$$

The overall objective of the present work was to systematically evaluate the efficacy of ferrous and alkaline activated persulfate to inactivate *E. coli* O157:H7 and *Listeria monocytogenes* in cell suspensions and identify the appropriate activation conditions. Specific objectives include: 1) to determine the effect of persulfate to activator ratios in pathogen inactivation; 2) to quantify free radical concentrations during persulfate activation; 3) to determine the major contributing radical in pathogen inactivation for each activation method.

3.2 Materials and methods

3.2.1 Chemicals

All chemicals were purchased from either Sigma-Aldrich (St. Louis, MO, USA) or Fisher Scientific (Fair Lawn, NJ, USA). The chemicals from Sigma-Aldrich include sodium persulfate, sodium thiosulfate, sodium chloride, sodium phosphate dibasic, anhydrous magnesium sulfate, benzoquinone (BQ), anisole and nitrobenzene (NB). The chemicals from Fisher Scientific include ferrous sulfate, ferric sulfate hydrate, sodium hydroxide solution (10 mol/L), sodium phosphate monobasic monohydrate, acetonitrile, isopropanol alcohol (IPA), and tert-butanol alcohol (TBA). All chemicals were at least reagent grade.

3.2.2 Bacterial cultures

Five strains of nalidixic acid-adapted *E. coli* O157:H7 and *L. monocytogenes* were obtained from USDA or University of Georgia Center for Food Safety (CFS). The *E. coli* O157:H7 strains were 1 (beef isolate), 4 (human isolate), 5 (human isolate), E009 (beef isolate),

and 932 (human isolate). The *L. monocytogenes* strains were Scott A (human isolate), LCDC 81-861 (raw cabbage isolate), F8027 (celery isolate), H77-50 (hot dog isolate), and F8369 (corn isolate). All strains were stored at -70 ^{o}C and recovered in tryptic soy broth (TSB, Difco, Sparks, MD, USA) for *E. coli* O157:H7 or brain heart infusion broth (BHIB, Difco, Sparks, MD, USA) for *L. monocytogenes* at 37 ^{o}C for 24 h. Then, a loop of each cultured broth was transferred to TSB supplemented with 50 mg/L nalidixic acid (TSBNA) or BHIB supplemented with 50 mg/L nalidixic acid (BHIBNA) and incubated at 37 ^{o}C for 24 h. The resulting cultures were confirmed by streaking onto sorbitol MacConkey agar (SMAC, Criterion, Santa Maria, CA, USA) for *E. coli* O157:H7 or Palcam Listeria selective agar (LSA, Bioworld, Dublin, OH, USA) supplemented with Palcam Listeria selective supplement (EMD Millipore Corporation, Billerica, MA, USA) for *L. monocytogenes* and incubated at 37 ^{o}C for 24 and 48 h, respectively. The same cultures were used as stock cultures after confirmation. Working cultures were made by sub-culturing the stock cultures twice in TSBNA (*E. coli* O157:H7) or BHIBNA (*L. monocytogenes*), each at 37 ^{o}C for 24 h. After sub-culture, the bacterial cells in the broth were harvested by centrifuging at 3,000 × g for 12 min. The cell pellets were washed with 20 mmol/L phosphate buffer saline (PBS, pH 7) once and re-suspended in PBS. Finally, equal volumes of each strain of each pathogen were mixed together to obtain a five-strain cocktail for the following experiment.

3.2.3 Activated persulfate treatment

One mL of prepared bacterial suspensions was added into sterile water containing a pre-calculated amount of sodium persulfate in a 150 mL sterile glass beaker. The beaker was placed on a magnetic stirrer plate and a magnetic bar spinning at high speed inside the beaker was used to keep the mixture homogenized. The treatment was initiated by adding a specific amount of activators (200 mmol/L ferrous sulfate or 1 mol/L sodium hydroxide). The total volume of the

mixture was 25 mL. At 60 s and 120 s, 1 mL was withdrawn from the mixture and transferred into 9 mL neutralizing buffer containing 5 g/L sodium thiosulfate and 20 mmol/L PBS for ferrous activation to neutralize the residual persulfate (Kambhu et al., 2012) or 100 mmol/L PBS for alkaline activation to neutralize the pH to quench the activation. Radical scavengers were also spiked into the activated persulfate mixtures to identify the major contributing radicals in pathogen inactivation. The radical scavengers used include IPA (scavengers for both hydroxyl and sulfate radicals, $k_{HO\cdot,IPA} = 1.9 \times 10^9$ L·mol^{-1}s^{-1}, $k_{SO_4^{-}\cdot,IPA} = 8.2 \times 10^7$ L·mol^{-1}s^{-1}), TBA (scavenger for hydroxyl radical only, $k_{HO\cdot,TBA} = 5.2 \times 10^8$ L·mol^{-1}s^{-1}, $k_{SO_4^{-}\cdot,TBA} < 10^6$ L·mol^{-1}s^{-1}), and BQ (scavenger for superoxide radical only, $k_{O_2^{-}\cdot,BQ} = 9.6 \times 10^8$ L·mol^{-1}s^{-1}) (Wu et al., 2014).

3.2.4 Microbiological analysis

The populations of each pathogen after the treatment were determined by plating 0.1 mL of 10-fold serial dilutions of the neutralized 10 mL mixtures onto tryptic soy agar supplemented with 0.1% sodium pyruvate (TSASP) for *E. coli* O157:H7 and brain heart infusion agar supplemented with 0.1% sodium pyruvate (BHIASP) for *L. monocytogenes* in duplicate. To determine samples with low populations, 0.25 mL of non-diluted samples were also plated in quadruplicate. The TSASP plates were incubated at 37 ºC for 24 h while the BHIASP plates were incubated at 37 ºC for 48 h before enumeration. Enrichment was also conducted by transferring 1 mL of the neutralized mixture into 9 mL of TSB supplemented with 0.1% sodium pyruvate (*E. coli* O157:H7) or BHIB supplemented with 0.1% sodium pyruvate (*L. monocytogenes*) for samples that showed no growth at the lowest dilutions. The addition of sodium pyruvate was to help recover injured cells. The broth was then incubated at 37 ºC for 24 h and streaked onto respective selective agars (SMAC or LSA). The detection limit of plating was 1.00 log CFU/mL. For samples that showed no growth on plates but positive after

enrichment, the population was deemed as 0.99 log CFU/mL while for samples showing negative after enrichment the population was deemed as 0.00 log CFU/mL.

3.2.5 Analysis of sulfate and hydroxyl radicals

Chemical probes anisole ($k_{HO\cdot,anisole} = 5.4 \times 10^9$ $L \cdot mol^{-1}s^{-1}$ and $k_{SO_4^{-}\cdot,anisole} = 4.9 \times 10^9$ $L \cdot mol^{-1}s^{-1}$) and NB ($k_{HO\cdot,NB} = 3.9 \times 10^9$ $L \cdot mol^{-1}s^{-1}$ and $k_{SO_4^{-}\cdot,NB} < 10^6$ $L \cdot mol^{-1}s^{-1}$) were used for quantification of steady-state concentrations of sulfate and hydroxyl radicals (Furman et al., 2011). The quantification was based on the degradation kinetics of two chemical probes under activated persulfate treatment. The concentration change of NB over time treated by activated persulfate can be expressed by Eq. 3.2. However, since the reaction rate of NB with hydroxyl radicals is 3 magnitudes faster than with sulfate radicals, the overall concentration change of NB can be simplified to Eq. 3.3, where $k_{obs,\,NB}$ is the observed NB degradation rate that follows pseudo-first-order kinetics. The degradation rate of anisole can be expressed in Eq. 3.4. Both $k_{obs,\,NB}$ and $k_{obs,anisole}$ can be calculated by plotting $Ln(C_t/C_0)$ over time, where C_t is the concentration of each chemical probe at time t and C_0 is the initial concentration. Thus, with $k_{obs,NB}$ and $k_{(HO\cdot,NB)}$ known, $[HO\cdot]_{ss}$ can be calculated with Eq. 3.3. When $k_{obs,anisole}$, $k_{obs,NB}$, $k_{(OH\cdot,NB)}$ and $[HO\cdot]_{ss}$ are known, $\left[SO_4^{-}\cdot\right]_{ss}$ can be calculated by Eq. 3.4. This radical quantification method using radical probes has been frequently reported in the literature (Ismail et al., 2017; Liang and Su, 2009; Wang et al., 2016).

$$\frac{d[NB]}{dt} = k_{OH\cdot,NB}[HO\cdot]_{ss}[NB] + k_{SO_4^{-}\cdot,NB}[SO_4^{-}\cdot]_{ss}[NB] \quad (3.2)$$

$$\frac{d[NB]}{dt} = k_{OH\cdot,NB}[HO\cdot]_{ss}[NB] = k_{obs,NB}[NB] \quad (3.3)$$

$$\frac{d[anisole]}{dt} = k_{OH\cdot,anisole}[HO\cdot]_{ss}[anisole] + k_{(SO_4^{\cdot-},\ anisole)}[SO_4^{\cdot-}]_{ss}[anisole] \quad (3.4)$$

$$= k_{obs,anisole}[anisole]$$

Anisole (100 µmol/L) and NB (100 µmol/L) were spiked together into activated persulfate solutions in all treatment conditions as described for the microbial treatment. For ferrous activation, 3 mL samples were withdrawn after 120 s treatment. For alkaline activation with low initial persulfate and sodium hydroxide concentrations, 3 mL samples were withdrawn after 4 and 8 h treatment. For alkaline activation with high initial persulfate and sodium hydroxide concentrations, 3 mL sample were withdrawn after 1.5 and 6 h treatment. All the withdrawn samples were mixed with 3 mL acetonitrile immediately to quench the reaction. The probes in the final 6 mL mixture were extracted by adding 0.3 g sodium chloride and 1.2 g anhydrous magnesium sulfate followed by centrifugation at 3000 × g for 5 min. After centrifugation, the upper organic phase was withdrawn and filtered through 0.2 µm nylon syringe filters (Restek, State College, PA, USA) into HPLC vials. A Shimadzu Prominence HPLC System (Shimadzu Scientific Instruments, Columbia, MD, USA) was used for chemical analysis. The chromatographic separation was carried out with a Supelco C18 reverse-phase column (25 cm × 4.6 mm, 5 µm particle size, Sigma-Aldrich, St. Louis, MO, USA). An isocratic mobile phase containing 60% acetonitrile and 40% ultrapure water with 0.1% formic acid at 0.8 mL/min was used. The chemical probes were detected with a Shimadzu SPD-M20A Prominence Diode Array Detector (Shimadzu Scientific Instruments, Columbia, MD, USA). Both anisole and NB were detected at 270 nm. Quantification of each chemical was based on five-point external standard curves.

3.2.6 Statistical analysis

All experiments related to microbial reductions were repeated three times while the experiment on free radicals were repeated two times. Means and standard deviations were calculated using Excel (Micro software, USA). Statistical analysis was conducted using SAS software 9.4 (SAS Institute Inc., Cary, NC, USA). One-way analysis of variance was conducted followed by Duncan's multiple range test with proc glm procedure. A $P < 0.05$ was considered to be significantly different.

3.3 Results and discussion

3.3.1 Effect of ferrous activated persulfate on pathogen inactivation

Table 3.1 shows the reductions of *E. coli* O157:H7 and *L. monocytogenes* treated by ferrous activated persulfate. The initial persulfate concentration was at 40 mmol/L and the persulfate to ferrous molar ratios was from 1:0.05 to 1:4. Ferric sulfate at 80 mmol/L was used as a control because ferric ion is the final activation product. The initial populations of *E. coli* O157:H7 and *L. monocytogenes* were 8.43 ± 0.05 and 8.64 ± 0.05 log CFU/mL, respectively. After 60 s treatment, 2.31 to 6.42 log CFU/mL reductions of *E. coli* O157:H7 were achieved depending on the persulfate to ferrous ratios. The maximum reduction was achieved at the ratio of 1:0.33. The ratio of 1:0.5 also showed statistically the same reductions (5.89 log CFU/mL). However, further increase of ferrous ratio to 1:1 resulted in significantly ($P < 0.05$) lower of *E. coli* O157:H7 reductions (5.18 log CFU/mL). The increase of ferrous ratio to 1:2 also lead to significantly ($P < 0.05$) lower *E. coli* O157:H7 reductions (2.31 CFU/mL) while further increase of ferrous ratio to 1:3 or 1:4 enhanced the reductions to about 4.00 log CFU/mL. Decrease of ferrous ratio from 1:0.33 to 1:05 also caused significantly ($P < 0.05$) less reductions. As a

comparison, only 1.25 log CFU/mL reductions of *E. coli* O157:H7 was achieved by ferric sulfate treatment in 60 s which was significantly ($P < 0.05$) lower than the reduction rates of *E. coli* O157:H7 treated by ferrous activated persulfate. The reduction trends of *E. coli* O157:H7 after 120 s treatment were similar to 60 s treatment but achieved greater reductions. The maximum reduction (7.77 log CFU/mL) after 120 s treatment was achieved at ratios of 1:0.33 and 1:0.5 while treatment at ratio 1:0.25 achieved lower but statistically not significant ($P \geq 0.05$) reductions (6.94 log CFU/mL). Further increase or decrease of the ratio resulted in significantly ($P < 0.05$) lower reductions of *E. coli* O157:H7 (Table 3.1).

Similar to *E. coli* O157:H7, ferrous activated persulfate showed the same effect on *L. monocytogenes*. The reductions of *L. monocytogenes* ranged from 0.79 to 6.34 log CFU/mL after 60 s treatment. Persulfate to ferrous ratio at 1:0.25 achieved the highest reductions. No significant ($P \geq 0.05$) differences of reductions were found when the ratio was changed to 1:0.33 and 1:0.5. However, further decrease and increase of ferrous ratios resulted in significantly ($P < 0.05$) lower reductions where 4.09 CFU/mL and 0.96 CFU/mL reductions were observed after 60 s treatment at ratios of 1:0.05 and 1:4, respectively. Ferric treatment showed only 0.76 log CFU/mL reductions after 60 s treatment. The extension of treatment to 120 s achieved higher reductions and the reduction trends were about the same as 60 s treatment. The highest reduction (7.25 log CFU/mL) was achieved at ratio 1:0.33 while similar reductions were achieved at ratios of 1:0.25 and 1:0.5. Furthermore, for both pathogens, although the extension of treatment time from 60 s to 120 s achieved more reductions, the improvements were generally slight, being around 1 log CFU/mL. In addition, both *E. coli* O157:H7 and *L. monocytogenes* were also treated individually by persulfate (40 mmol/L) and ferrous sulfate (160 mmol/L), and no significant reductions were found (data not shown).

The general persulfate activation reaction using ferrous is shown in Eq. 3.5. Ferrous ion is oxidized to ferric ion while persulfate ion is reduced to sulfate ion and sulfate radical (Liang et al., 2008). The generated sulfate radicals can further react with water molecules to form hydroxyl radicals (Eq. 3.6). Thus, the major free radicals produced by ferrous activated persulfate are sulfate and hydroxyl radicals. As shown in Table 3.1, the persulfate to ferrous ratio played an important role in determining the inactivation efficacy. Our results showed the persulfate to ferrous ratio at 1:0.33 achieved the highest reduction in 120 s for both pathogens. As reported in the literature, an appropriate amount of ferrous is needed to maximize the persulfate activation process (Matzek and Carter, 2016). Insufficient ferrous will result in inefficient usage of persulfate and less sulfate radical can be produced (Eq. 3.5). Excess ferrous is also not appropriate because ferrous can act as sulfate radical scavengers (Eq. 3.7) to reduce the overall available sulfate radicals. This explains why further increase or decrease of ferrous ratio from the optimum (around 1:0.33 in our data) resulted in decreased inactivation efficacy (Table 3.1). Liang et al. (2004b) found the degradation of trichloroethylene by ferrous activated persulfate was maximized at the ratio of 1:1 while further increase of ferrous reduced the overall degradation efficacy. Wordofa et al. (2017) also observed that the increase of ferrous concentration from 3 to 6 mmol/L in persulfate activation resulted in slight decreases of the viability loss rate constant of *E. coli* O157:H7.

$$S_2O_8^{2-} + Fe^{2+} \rightarrow SO_4^{-}\cdot + SO_4^{2-} + Fe^{3+} \quad (3.5)$$

$$SO_4^{-}\cdot + H_2O \rightarrow SO_4^{2-} + OH\cdot + H^{+} \quad (3.6)$$

$$SO_4^{-}\cdot + Fe^{2+} \rightarrow Fe^{3+} + SO_4^{2-} \quad (3.7)$$

The steady-state concentrations of free radicals were measured to further confirm and explain the persulfate to ferrous ratio effect on the overall pathogen inactivation efficacy. Table

3.1 shows the measured hydroxyl and sulfate radicals produced by ferrous activated persulfate at each condition. In ferrous activated persulfate, $[HO\cdot]_{ss}$ ranged from 1.13×10^{-13} to 3.16×10^{-14} mol/L and $[SO_4^{-}\cdot]_{ss}$ ranged from 1.30×10^{-13} to 2.99×10^{-14} mol/L. The change of free radical concentration trends coincided with the microbial reduction results that high free radical concentrations were found at the activation conditions showing high microbial reductions. The free radical data confirmed our assumption that persulfate to ferrous ratio at 1:0.33 provided maximum activation because this condition showed the highest free radical concentrations. As the ratio changed, the concentration of free radicals decreased and resulted in decreased pathogen reductions.

It was also noticed that *L. monocytogenes* was less susceptible to ferrous activated persulfate treatment compared with *E. coli* O157:H7. This might be due to the differences in the cell wall and cell membrane structures between Gram-negative and Gram-positive bacteria. Similar results were found by Barnes et al. (2013) that *Staphylococcus aureus* was more resistant than *E. coli* against the reactive oxygen species produced by UV light (365 nm) activated TiO_2 in a 1-h treatment. Similarly, Kim et al. (2013) demonstrated *L. monocytogenes* was more resistant than *E. coli* and *Salmonella* typhimurium when treated with UVC light activated TiO_2. Zhang et al. (2018) also found the vanadium tetrasulfide-based photocatalytic disinfection was more effective on Gram-negative bacteria than on Gram-positive bacteria. They suggested such differences were due to the cell wall structure differences as the thicker cell wall of Gram-positive bacteria consisting peptidoglycan and teichoic acids can work as a natural barrier and limit the disinfectant attack. The cell wall of Gram-negative bacteria, on the other hand, is thinner and thus are more sensitive to radical attack even with the presence of an outer membrane (Zhang et al., 2018). However, Yadav et al. (2014) found contradictory results where

visible light activated Ni-TiO_2 nanoparticles were more effective in inactivating Gram-Positive bacteria than Gram-negative bacteria. They speculated this phenomenon is caused by the different affinities between cell envelope structures and photo-catalyst as the outer cell membrane of Gram-negative bacteria tend to restrict the adsorption of the active catalyst. Nakamura et al. (2015) had similar conclusions but different results. They found Gram-negative bacteria were more sensitive to photo-irradiated poly-phenol treatment than Gram-positive bacteria and they ascribed the results to the higher affinity between Gram-negative bacteria and polyphenols.

3.3.2 Effect of alkaline activated persulfate on pathogen inactivation

Sodium hydroxide was used as the alkaline reagent to activate persulfate for pathogen inactivation. For *E. coli* O157:H7, the initial persulfate concentration was set at 40 mmol/L and the sodium hydroxide concentration was changed from 5 to 30 mmol/L to achieve different alkalinity. The results (Table 3.2) showed that by increasing the sodium hydroxide concentrations, the reductions achieved in the first 60 s increased from 0.57 to 5.68 CFU/mL. Significant reduction of *E. coli* O157:H7 was not observed until the sodium hydroxide concentration was increased to 10 mmol/L. *E. coli* O157:H7 suspensions were also treated with sodium hydroxide alone. Persulfate activated by sodium hydroxide always achieved significantly ($P < 0.05$) higher reductions than sodium hydroxide alone at each comparable alkalinity level (except at 5 mmol/L sodium hydroxide). The increase of treatment time from 60 s to 120 s achieved more reductions for both activated persulfate treatment and sodium hydroxide alone treatment with the same reduction trends. However, the improvement was generally not significant. The highest reduction (6.21 log CFU/mL) was achieved by persulfate activated with 30 mmol/L sodium hydroxide.

The same alkaline activation conditions used for *E. coli* O157:H7 did not achieve significant efficacy on *L. monocytogenes* (data not shown). Thus, a higher initial persulfate concentration (500 mmol/L) was used combined with a higher amount of initial sodium hydroxide. After 60 s treatment, 0.54 to 3.77 log CFU/mL reductions of *L. monocytogenes* were observed (Table 3.3). Higher reductions were achieved by increasing the initial sodium hydroxide concentrations. Similar to *E. coli* O157:H7, persulfate activated by sodium hydroxide achieved significantly ($P < 0.05$) more reductions of *L. monocytogenes* compared to sodium hydroxide treatment alone at each comparable alkalinity level. However, different than *E. coli* O157:H7, the extension of treatment from 60 s to 120 s resulted in dramatic increase of *L. monocytogenes* reductions where about 5 more log CFU/mL reductions were achieved for persulfate activated by 200 to 350 mmol/L sodium hydroxide. Complete inactivation (8.64 log CFU/mL reductions) of *L. monocytogenes* (no growth after enrichment) was achieved after 120 s treatment when 350 mmol/L sodium hydroxide was used for activation.

The activation reaction of persulfate by alkaline reagent is shown in Eq. 3.8 (Furman et al., 2010). Persulfate is hydrolyzed under alkaline pH to produce sulfate and superoxide radicals. Sulfate radicals can further react with hydroxide ions to produce hydroxyl radicals. Therefore, there are three major free radicals in alkaline activated persulfate. Our results showed persulfate activated by sodium hydroxide was significantly ($P < 0.05$) more effective in inactivating both pathogens at each comparable level than sodium hydroxide alone except at 5 mM and 100 mM sodium hydroxide. This indicated enough sodium hydroxide is needed for persulfate activation in order to achieve high antimicrobial efficacy. This is in accordance with the study of Furman et al. (2011) who revealed the reactivity of alkaline activated persulfate would increase with increasing basicity. It also indicated the activation product contributed significantly in pathogen

activation, especially for *L. monocytogenes*, where at least 3 more log CFU/mL reductions were achieved by combining persulfate and sodium hydroxide than sodium hydroxide alone (Table 3.3).

$$2S_2O_8^{2-} + 2H_2O \xrightarrow{OH^-} 3SO_4^{2-} + SO_4^{-}\cdot + O_2^{-}\cdot + 4H^+ \quad (3.8)$$

$$SO_4^{-}\cdot + OH^- \rightarrow SO_4^{2-} + OH\cdot \quad (3.9)$$

The steady-state concentrations of hydroxyl and sulfate radicals were also measured as shown in Tables 3.2-3 . In sodium hydroxide activated persulfate, $[HO\cdot]_{ss}$ ranged from 9.05×10^{-16} to 5.02×10^{-16} mol/L (Table 3.2) with 40 mmol/L initial persulfate and from 1.48×10^{-14} to 5.44×10^{-15} mol/L (Table 3.3) with 500 mmol/L initial persulfate; $[SO_4^{-}\cdot]_{ss}$ ranged from 5.95×10^{-15} to 8.57×10^{-16} mol/L (Table 3.2) with 40 mmol/L initial persulfate and from 3.69×10^{-14} to 2.66×10^{-15} mol/L (Table 3.3) with 500 mmol/L initial persulfate. Higher sodium hydroxide levels lead to lower concentrations of both sulfate and hydroxyl radicals. This might be because elevated alkalinity can convert more sulfate radicals into hydroxyl radicals (Eq. 3.9) and also expedite the self-decomposition process of hydroxyl radicals. In addition, lower hydroxyl and sulfate radical concentrations were found on activation conditions showing higher microbial reductions, indicating both hydroxyl and sulfate radicals might not be the major contributing radical in pathogen inactivation. Superoxide radicals (Eq. 3.8) might be the major radical in alkaline activated persulfate for pathogen inactivation. However, we were not able to quantify superoxide radical due to the lack of reliable chemical probes.

3.3.3 Determination of major contributing radical in pathogen inactivation

In order to determine the major radical in pathogen inactivation, *E. coli* O157:H7 and *L. monocytogenes* were treated by activated persulfate spiked with or without radical scavengers. Fig. 3.1 shows the inactivation results for ferrous activated persulfate. Persulfate at initial

concentration of 40 mmol/L activated with 13.3 mmol/L ferrous sulfate was used. For both pathogens, ferrous activated persulfate treatment spiked with radical scavengers resulted in significantly ($P < 0.05$) lower reductions (more than 5 log CFU/mL difference) compared with that without radical scavengers, indicating free radicals were the major compounds that inactivated both pathogen cells. For *E. coli* O157:H7, isopropanol alcohol (IPA, scavengers for both sulfate and hydroxyl radicals) spiked treatment showed significantly ($P < 0.05$) lower reductions than that of tert-butanol alcohol (TBA, scavenger for hydroxyl radical) spiked treatment. However, the absolute differences were only about 1 log CFU/mL. For *L. monocytogenes*, no significant ($P \geq 0.05$) differences were found between IPA and TBA spiked treatment. This may indicate hydroxyl radical was the major radical of ferrous activated persulfate in inactivating *E. coli* O157:H7 and *L. monocytogenes*. This is contradictory to the conclusiosn of the study by Wordofa et al. (2017) that argued sulfate radical was the major radical of ferrous activated persulfate in *E. coli* O157:H7 inactivation. They reached this conclusion using theoretical models that ascertained significantly more sulfate radicals were generated than hydroxyl radicals by ferrous activated persulfate. However, they did not verify their conclusion with radical scavengers or radical qualification and quantification approaches, making their statement less convincing.

Zou et al. (2013) and Dulova et al. (2017) have indicated that both hydroxyl and sulfate radicals can be generated by ferrous activated persulfate and they contributed equally in chemical degradations. In our study, although similar amount of hydroxyl and sulfate radicals were produced (Table 3.1), hydroxyl radical was found to contribute the most in pathogen inactivation through radical scavenger treatment (Fig 1). This might be because hydroxyl radical is a nonselective oxidant while sulfate radical is a selective oxidant and prone to react with

electron-rich chemicals (Neta et al., 1977; Wordofa et al., 2017). The antimicrobial activity of hydroxyl radicals has been demonstrated in recent studies. Nakamura et al. (2016) found hydroxyl radicals produced by hydrogen peroxide photolysis could achieve more than 5 log reductions of *Streptococcus mutans* on biofilms (24-h-old). Hydroxyl radical was also considered to be the major compound that provided the synergistic effect on antibiofilm activity when combining pleurocidin and antibiotics (Choi and Lee, 2012).

On the other hand, Fig. 3.2 shows the results for sodium hydroxide activated persulfate treatment spiked with or without radical scavengers. For *E. coli* O157:H7, although IPA and TBA spiked treatment showed significantly ($P < 0.05$) lower reductions compared with the treatment without scavengers, the differences were only about 1.5 log CFU/mL. For *L. monocytogenes*, IPA and TBA spiked treatment also did not result in much lower reductions compared with the non-scavenger control. However, treatment spiked with benzoquinone (BQ, scavengers for superoxide radicals) resulted in significantly ($P < 0.05$) lower reductions for both *E. coli* O157:H7 (more than 7 log CFU/mL difference) and *L. monocytogenes* (more than 5 log CFU/mL difference) compared with the non-scavenger controls. These indicated superoxide radical may be the major contributing radical of sodium hydroxide activated persulfate in pathogen inactivation. This is in agreement with the results of Liang et al. (2015) and Zhang et al. (2018) who demonstrated superoxide radicals were the major contributors in bacteria inactivation in their advanced oxidation process systems and are more effective than hydroxyl radicals.

3.3.4 Comparison between ferrous and alkaline activated persulfate

In order to measure the activation process for ferrous and alkaline activated persulfate, two radical probes, anisole (reactive with sulfate and hydroxyl radicals) and nitrobenzene (NB,

reactive with hydroxyl radical), were spiked into activated persulfate solution and the degradation curves were recorded (Fig. 3.3). For ferrous activated persulfate (Fig. 3.3-A), both probe curves went through an instant drop at the beginning (first 2 min) then the concentration of NB remained constant and anisole went through a slow degradation afterward. For alkaline activated persulfate (Fig. 3.3-B&C), both probes showed linear degradation trends with time. It was obvious that ferrous activation only lasted for a short period of time as the degradation of NB stopped and the degradation of anisole was dramatically slowed after 2 min activation (Fig. 3.1-A). This indicated no hydroxyl radical existed and only slight amount of sulfate radical was being produced after the initial activation. This was due to the fast reaction between ferrous and persulfate that resulted in fast depletion of both chemicals. Liang et al. (2004a) reported similar findings that the degradation of trichloroethylene by ferrous activated persulfate stalled after only a few minutes. At acidic conditions, persulfate can be slowly hydrolyzed into peroxymonosulfate and hydrogen peroxide (Eq. 3.10-12) (Mariano, 1968). Hydrogen peroxide can reduce ferric ion back to ferrous ion and continue to activate the remaining persulfate. However, this process is so slow that only negligible hydroxyl radical (Eq. 3.6) can be produced. That is why we observed a slow degradation curve of anisole in Fig. 3.1-A.

$$S_2O_8^{2-} + H_3O^+ \leftrightarrow HS_2O_8^- + H_2O \quad (3.10)$$

$$HS_2O_8^- \rightarrow HSO_5^- + SO_3 \quad (3.11)$$

$$HSO_5^- + H_2O \rightarrow HSO_4^- + H_2O_2 \quad (3.12)$$

On the other hand, the persulfate activation by sodium hydroxide can last much longer. As shown in Fig. 3.1-B&C, continuous degradation of anisole and NB were observed, indicating continuous production of sulfate and hydroxyl radicals as well as superoxide radicals. Therefore, alkaline activated persulfate is advantageous against ferrous activated persulfate due to the

longer activation period, which makes it more suitable for repeated use in produce sanitation. However, ferrous activation can be improved by continuous addition or sequential addition of small amount of ferrous to extend the activation period and achieve higher efficacies. For example, Liang et al. (2004a) achieved improved trichloroethylene degradation by adding ferrous sequentially into the persulfate solution. Therefore, we are assuming this sequential ferrous addition method can improve the overall pathogen inactivation efficacy. Our next research step will be aiming to study the sequential addition effect, initial persulfate concentration effect, and the inactivation mechanism.

3.4 Conclusion

The results of present study showed sodium persulfate activated by ferrous sulfate and sodium hydroxide can effectively inactivate *E. coli* O157: H7 and *L. monocytogenes*. The persulfate to activator molar ratios played an important role in determining the overall pathogen inactivation efficacy. For ferrous activation, insufficient or excess amount of ferrous sulfate will lead to reduced pathogen inactivation efficacies. For alkaline activation, the inactivation efficacy can be improved by higher basicity. Hydroxyl radical was determined to be the major contributing radical in ferrous activation while superoxide radical was determined to be the major contributing radical in alkaline activation to inactivate *E. coli* O157: H7 and *L. monocytogenes*. Alkaline activation can be advantageous to ferrous activation due to the prolonged activation period. Based on our results, activated persulfate could be used as a novel sanitizer on fresh produce sanitation.

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Table 3.1. Efficacy of ferrous activated persulfate in inactivating *E. coli* O157:H7 and *L. monocytogenes* at different persulfate to ferrous molar ratios and the measured steady-state concentrations of hydroxyl and sulfate radicals.

PS to ferrous molar ratios	Reduction (log CFU/mL)				Concentration (mol/L)	
	E.coli O157:H7		*L. monocytogenes*			
	(60 s)	(120 s)	(60 s)	(120 s)	$[HO^{\bullet}]_{ss}$	$[SO_4^{-\bullet}]_{ss}$
40 mmol/L PS + 2 mmol/L Fe^{2+} (1:0.05)	4.00 ± 1.25 C	4.45 ± 0.99 C	4.09 ± 0.15 C	4.26 ± 0.27 C	3.16×10^{-14}	3.83×10^{-14}
40 mmol/L PS + 4 mmol/L Fe^{2+} (1:0.1)	5.12 ± 0.59 B	5.96 ± 0.47 C	4.04 ± 0.71 C	5.15 ± 0.90 B	3.75×10^{-14}	4.53×10^{-14}
40 mmol/L PS + 10 mmol/L Fe^{2+} (1:0.25)	5.38 ± 0.21 B	6.94 ± 0.43 AB	6.34 ± 0.36 A	6.89 ± 0.39 A	9.40×10^{-14}	1.30×10^{-13}
40 mmol/L PS + 13.3 mmol/L Fe^{2+} (1:0.33)	6.42 ± 0.41 A	7.77 ± 0.57 A	6.23 ± 0.08 A	7.25 ± 0.36 A	1.13×10^{-13}	1.17×10^{-13}
40 mmol/L PS + 20 mmol/L Fe^{2+} (1:0.5)	5.89 ± 0.41 AB	7.77 ± 0.57 A	6.06 ± 0.27 A	7.09 ± 0.59 A	1.13×10^{-13}	9.14×10^{-14}
40 mmol/L PS + 40 mmol/L Fe^{2+} (1:1)	5.18 ± 0.45 B	6.62 ± 0.35 BC	4.87 ± 0.69 B	5.72 ± 0.70 B	1.03×10^{-13}	6.52×10^{-14}
40 mmol/L PS + 80 mmol/L Fe^{2+} (1:2)	2.31 ± 0.24 D	2.34 ± 0.34 E	1.01 ± 0.11 D	2.25 ± 0.10 D	5.99×10^{-14}	2.99×10^{-14}
40 mmol/L PS + 120 mmol/L Fe^{2+} (1:3)	3.94 ± 0.06 C	4.00 ± 0.14 D	0.79 ± 0.03 D	1.68 ± 0.06 D	6.41×10^{-14}	4.23×10^{-14}
40 mmol/L PS + 160 mmol/L Fe^{2+} (1:4)	4.00 ± 0.04 C	4.19 ± 0.18 D	0.96 ± 0.03 D	2.02 ± 0.11 D	3.72×10^{-14}	3.90×10^{-14}
80 mmol/L Fe^{3+}	1.25 ± 0.22 E	1.34 ± 0.17 F	0.76 ± 0.03 D	1.43 ± 0.40 D		

The initial populations were 8.43 ± 0.05 log CFU/mL (*E. coli* O157:H7) and 8.64 ± 0.05 (*L. monocytogenes*). Persulfate was set at 40 mmol/L in all treatment conditions. Means (n = 3) in the same column followed by the same capital letters are not significantly different ($P \geq 0.05$). PS = persulfate. $[\ ]_{ss}$ indicates the steady-state concentration of the free radical.

Table 3.2. Efficacy of alkaline activated persulfate in inactivating *E. coli* O157:H7 at different sodium hydroxide levels and the measured steady-state concentrations of hydroxyl and sulfate radicals.

Treatment conditions	Reduction (log CFU/mL)		Concentration (mol/L)	
	(60 s)	(120 s)	$[HO\cdot]_{ss}$	$[SO_4^{-}\cdot]_{ss}$
40 mmol/L PS + 5 mmol/L NaOH	0.57 ± 0.06 DE	0.58 ± 0.04 E	8.21×10^{-16}	5.95×10^{-15}
40 mmol/L PS + 10 mmol/L NaOH	3.10 ± 0.62 C	4.15 ± 0.39 C	9.05×10^{-16}	5.36×10^{-15}
40 mmol/L PS + 15 mmol/L NaOH	4.32 ± 0.43 B	4.66 ± 0.56 BC	7.61×10^{-16}	3.02×10^{-15}
40 mmol/L PS + 20 mmol/L NaOH	4.61 ± 0.42 B	5.13 ± 0.22 B	6.96×10^{-16}	8.77×10^{-16}
40 mmol/L PS + 25 mmol/L NaOH	4.95 ± 0.07 B	5.92 ± 0.78 A	5.55×10^{-16}	9.26×10^{-16}
40 mmol/L PS + 30 mmol/L NaOH	5.68 ± 0.69 A	6.21 ± 0.87 A	5.02×10^{-16}	8.57×10^{-16}
5 mmol/L NaOH	0.08 ± 0.13 E	0.17 ± 0.10 E		
10 mmol/L NaOH	0.40 ± 0.18 E	0.32 ± 0.27 E		
15 mmol/L NaOH	0.59 ± 0.04 DE	0.71 ± 0.06 E		
20 mmol/L NaOH	1.16 ± 0.05 D	1.97 ± 0.54 D		
25 mmol/L NaOH	2.66 ± 0.66 C	4.49 ± 0.55 BC		
30 mmol/L NaOH	4.42 ± 0.19 B	5.07 ± 0.19 B		

The initial *E. coli* O157:H7 population was 8.37 ± 0.05 log CFU/mL. Means (n = 3) in the same column followed by the same capital letters are not significantly different ($P \geq 0.05$). PS = persulfate. $[\ ]_{ss}$ indicates the steady-state concentration of the free radical.

Table 3.3. Efficacy of alkaline activated persulfate in inactivating *L. monocytogenes* at different sodium hydroxide levels and the measured steady-state concentrations of hydroxyl and sulfate radicals.

Treatment conditions	Reduction (log CFU/mL)		Concentration (mol/L)	
	(60 s)	(120 s)	$[HO\cdot]_{ss}$	$[SO_4^{-}\cdot]_{ss}$
500 mmol/L PS + 100 mmol/L NaOH	0.54 ± 0.14 E	0.92 ± 0.06 EF	1.48×10^{-14}	3.69×10^{-14}
500 mmol/L PS + 150 mmol/L NaOH	1.41 ± 0.24 C	2.99 ± 0.78 D	1.21×10^{-14}	7.17×10^{-15}
500 mmol/L PS + 200 mmol/L NaOH	2.84 ± 0.55 B	7.44 ± 0.35 B	9.62×10^{-15}	3.82×10^{-15}
500 mmol/L PS + 250 mmol/L NaOH	2.71 ± 0.24 B	7.65 ± 0.00 B	8.74×10^{-15}	2.66×10^{-15}
500 mmol/L PS + 300 mmol/L NaOH	2.87 ± 0.18 B	7.65 ± 0.00 B	5.78×10^{-15}	4.99×10^{-15}
500 mmol/L PS + 350 mmol/L NaOH	3.77 ± 0.22 A	8.64 ± 0.00 A	5.44×10^{-15}	4.02×10^{-15}
100 mmol/L NaOH	0.19 ± 0.05 E	0.27 ± 0.05 G		
150 mmol/L NaOH	0.26 ± 0.03 E	0.33 ± 0.05 G		
200 mmol/L NaOH	0.30 ± 0.07 E	0.43 ± 0.18 FG		
250 mmol/L NaOH	0.51 ± 0.11 E	1.25 ± 0.20 E		
300 mmol/L NaOH	0.97 ± 0.33 D	3.13 ± 0.50 D		
350 mmol/L NaOH	2.71 ± 0.26 B	5.44 ± 0.27 C		

The initial *L. monocytogenes* population was 8.64 ± 0.05 log CFU/mL. Means (n = 3) in the same column followed by the same capital letters are not significantly different ($P < 0.05$). PS = persulfate. $[\ ]_{ss}$ indicates the steady-state concentration of the free radical.

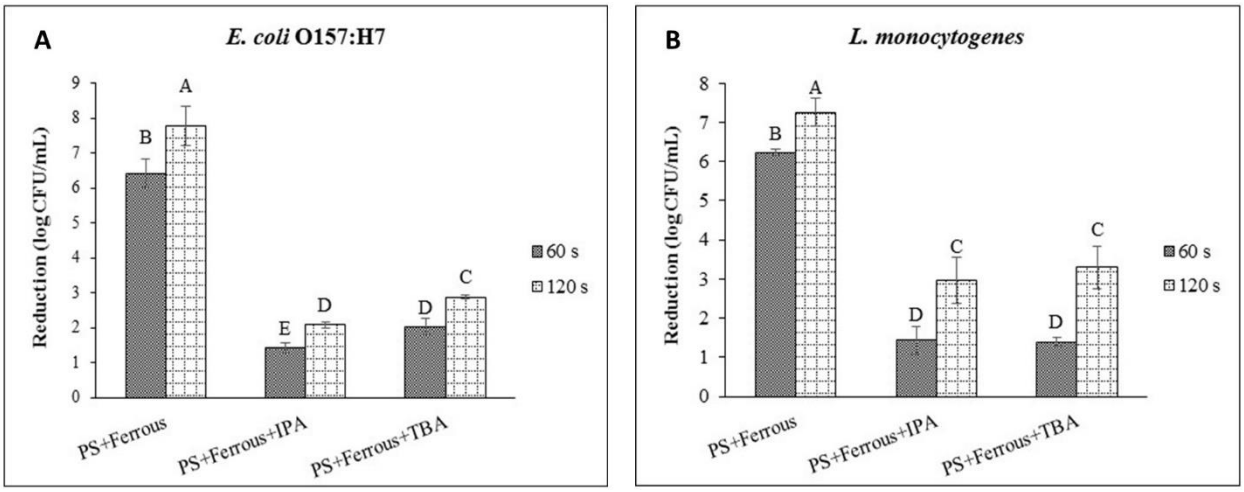

Fig. 3.1. Inactivation of *E. coli* O157:H7 (A) and *L. monocytogenes* (B) by ferrous activated persulfate spiked with or without radical scavengers. The initial persulfate concentration was at 40 mmol/L and the initial concentration of ferrous sulfate was at 13.3 mM. The initial IPA and TBA concentrations were 0.5 mol/L. Columns with the same capital letters are not significantly different ($P \geq 0.05$). PS = persulfate. IPA = isopropanol alcohol. TBA = tert-butanol alcohol.

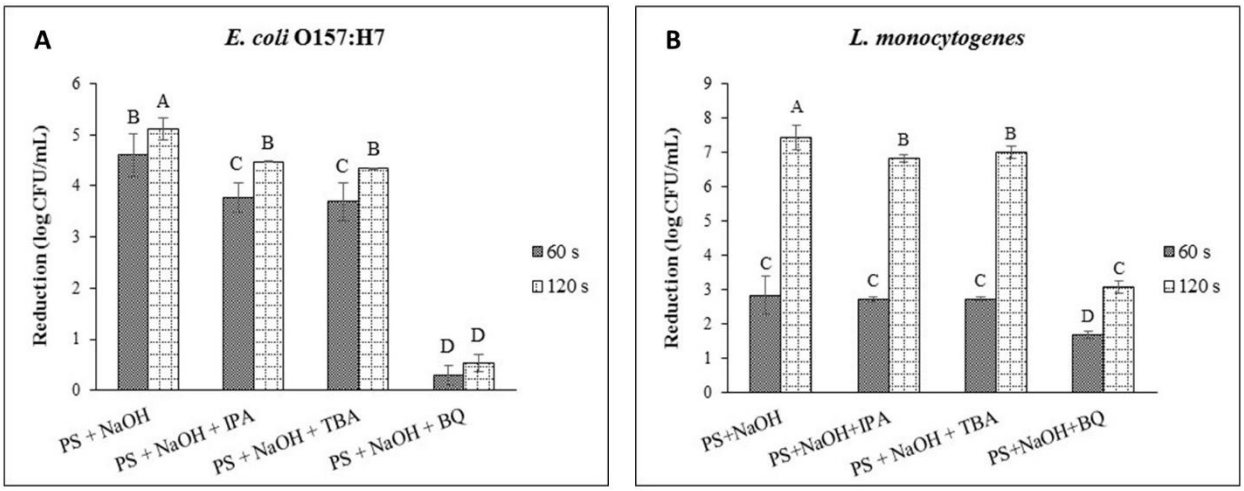

Fig. 3.2. Inactivation of *E. coli* O157:H7 (A) and *L. monocytogenes* (B) by alkaline activated persulfate spiked with or without radical scavengers. The initial persulfate concentrations were 40 mmol/L for *E. coli* O157:H7 and 500 mmol/L for *L. monocytogenes*. The initial sodium hydroxide concentrations were at 20 mmol/L for *E. coli* O157:H7 and 200 mmol/L for *L. monocytogenes.* The initial IPA and TBA concentrations were 0.5 mol/L and the initial BQ concentration was 10 mmol/L. Columns with the same capital letters are not significantly different ($P \geq 0.05$). PS = persulfate. IPA = isopropanol alcohol. TBA = tert-butanol alcohol. BQ = benzoquinone.

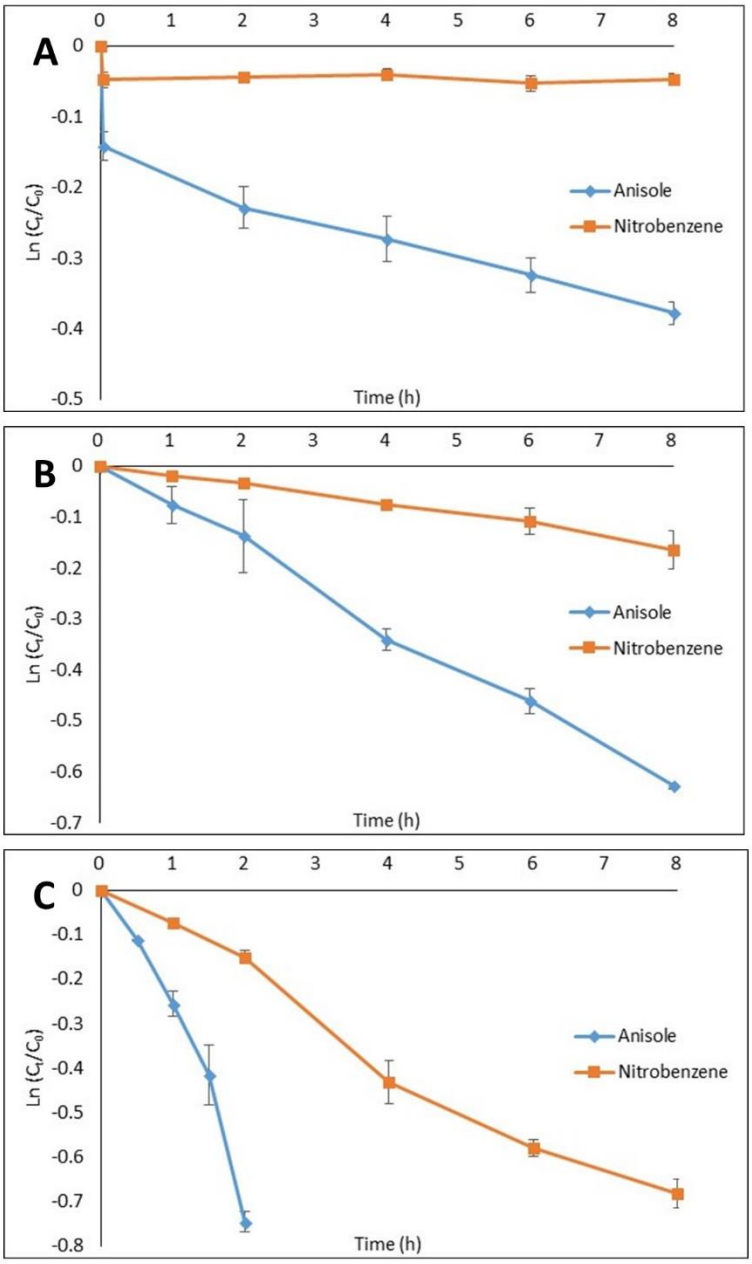

Fig. 3.3. Degradation of anisole and nitrobenzene when subjected to: A) 40 mmol/L PS + 13.3 Fe^{2+}; B) 40 mmol/L PS + 20 mmol/L NaOH; C) 400 mmol/L PS + 200 mmol/L NaOH. PS = persulfate.

CHAPTER 4

EFFICACY OF ACTIVATED PERSULFATE IN PATHOGEN INACTIVATION: A FURTHER EXPLORATION

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Abstract

Activated persulfate, a relatively new advanced oxidation process, has gained attention for its potential to ensure fresh produce safety. One of the major advantages is to avoid the formation of toxic chlorinated disinfection by-products which are concerns for chlorine-based sanitizers. This study aimed to investigate the efficacy of ferrous and alkaline activated persulfate in inactivating *Escherichia coli* O157:H7 and *Listeria monocytogenes*, with the primary focus on the effect of initial persulfate concentration, the effect of gradual addition of ferrous ion, and the stability of activated persulfate. The prepared 5-strain pathogen cocktails were treated by activated persulfate for 60 or 120 s. Sodium thiosulfate combined with phosphate buffer was used to quench the reaction. Both pathogens were plated onto non-selective agars for colony enumeration. The steady-state concentrations of sulfate and hydroxyl radicals were quantified in each activation condition. The results showed higher initial persulfate concentration can lead to more pathogen reductions. About 8.50 log CFU/mL reduction was observed in 120 s after the initial persulfate concentration was increased to 80 mmol/L (ferrous activation on both pathogens) or 600 mmol/L (alkaline activation on *L. monocytogenes*). Gradual addition of ferrous ion into persulfate solution achieved more pathogen reductions than adding all ferrous ion at once. However, only the increases in reduction achieved by four sequential addition were significant ($P < 0.05$). In addition, the steady-state concentrations of both sulfate and hydroxyl radicals were found to be positively correlated with microbial reductions at all conditions. Furthermore, the pathogen inactivation efficacy of both ferrous and alkaline activated persulfate can be maintained for a relatively long period (up to 3 h).

Keywords

Advanced oxidation process; free radicals; *Escherichia coli* O157:H7; *Listeria monocytogenes*; sanitizer

4.1 Introduction

Fresh produce are potential carriers for foodborne pathogens and can lead to significant illness upon consumption (Olmez & Kretzschmar, 2009). Painter et al. (2013) indicated majority of foodborne illnesses are caused by consumption of fresh leafy vegetables. In the fresh produce industry, one of the key steps is to apply appropriate processing treatment to effectively remove and inactivate potential foodborne pathogens on produce (Joshi, Mahendran, Alagusundaram, Norton, & Tiwari, 2013; Olaimat & Holley, 2012). Currently, chlorine-based sanitizers are most commonly used for produce washing and sanitation in the U.S. fresh produce industry (Feliziani, Lichter, Smilanick, & Ippolito, 2016). However, chlorine can react with organic matter from fresh produce and generate toxic disinfection by-products (DBPs). Once consumed, those DBPs can cause adverse health effect, including both acute and chronic illness (Meireles, Giaouris, & Simoes, 2016). Therefore, an alternative sanitizer that will produce less or no toxic DBPs is needed. Many alternative sanitizers and processing technologies have been developed and studied for produce sanitation, including electrolyzed water (Gil, Gomez-Lopez, Hung, & Allende, 2015), peracetic acid (Singh, Hung, & Qi, 2018), ozone (Khadre, Yousef, & Kim, 2001), chlorine dioxide (Tomás-Callejas et al., 2012), and physical-based treatment (e.g. ionizing irradiation) (Meireles et al., 2016). As a comparison, advanced oxidation process (AOP), a free radical-based technology, has not been fully explored for its potential application in fresh produce sanitation.

AOP is commonly used for wastewater treatment and can be applied at ambient temperature and normal pressure with *in-situ* generation of highly oxidative and reactive radicals, such as hydroxyl radical (Oturan & Aaron, 2014). AOP can be more advantageous than other technologies due to its fast reaction rates and non-selective oxidation power

(Antonopoulou, Evgenidou, Lambropoulou, & Konstantinou, 2014). Many reacting systems belong to AOP, such as chemical-based AOP (*e.g.* Fenton's reagent and peroxonation) (Oturan & Aaron, 2014), photochemical-based AOP (*e.g.* UV/H_2O_2 and UV/TiO_2) (Hernandez, Zappi, Colucci, & Jones, 2002; Mills & Le Hunte, 1997), sonochemical-based AOP (e.g. ultrasound/water) (Ghodbane & Hamdaoui, 2009), and electrochemical-based AOP (Sirés & Brillas, 2012). These AOP-based technologies and treatments have been demonstrated to be effective in degrading many chemical pollutants (Oturan & Aaron, 2014). On the other hand, although some studies have demonstrated AOP-based treatment can effectively inactivate microbial cells (Kim et al., 2013; Pokhum, Viboonratanasri, & Chawengkijwanich, 2017; Zhang, Zou, Cai, Li, & He, 2018), information regarding applying AOP-based technologies on pathogenic microbe inactivation are still limited.

Activated persulfate ($S_2O_8^{2-}$) is a relatively new chemical-based AOP and has shown potential for microbial inactivation. Persulfate can be activated by different activators, such as UV light, transition metals, heat, ultrasound, and alkaline pH (Matzek & Carter, 2016). Studies have demonstrated its efficacy for environmental water disinfection against several microbes, including phytoplankton (Samyoung, Peterson, Righter, Miles, & Tratnyek, 2013), *Microcystis aeruginosa* (Wang, Chen, Xie, Shang, & Ma, 2016), and non-pathogenic *Escherichia coli* (Garkusheva et al., 2017). Recent studies also demonstrated UV-C light and ferrous ion activated persulfate can effectively inactivate pathogenic *E. coli* in environmental water samples (Sun, Corey, & Huang, 2016; Wordofa, Walker, & Liu, 2017). However, all these studies focused on environmental sample disinfection with a relatively long period of treatment time (hours and days treatment), which is not practical for produce sanitation. To our best knowledge, our previous study (Qi, Huang, & Hung, 2018) was the first study that tried to apply activated

persulfate for produce sanitation using a much shorter treatment time (within minutes). The efficacy of ferrous (Eq. 4.1&2) and alkaline (Eq. 4.3&4) activated persulfate in inactivating foodborne pathogens was evaluated and high pathogen reduction (more than 7 log CFU/mL) could be achieved in minutes. The optimum persulfate to activator ratio and the major contributing radical in pathogen inactivation were also deduced. However, a lot more information remains unclear. In addition, since the pathogen inactivation efficacy of activated persulfate is mainly dependent on the free radicals and no chlorine is involved, toxic chlorine-based DBPs can be minimized.

$$S_2O_8^{2-} + Fe^{2+} \rightarrow SO_4^{-}\cdot + SO_4^{2-} + Fe^{3+} \quad (4.1)$$

$$SO_4^{-}\cdot + H_2O \rightarrow SO_4^{2-} + OH\cdot + H^{+} \quad (4.2)$$

$$S_2O_8^{2-} + 2H_2O \xrightarrow{OH^-} SO_4^{2-} + SO_4^{-}\cdot + O_2^{-}\cdot + 4H^{+} \quad (4.3)$$

$$SO_4^{-}\cdot + OH^{-} \rightarrow SO_4^{2-} + OH\cdot \quad (4.4)$$

The current study continued from our previous study (Qi et al., 2018) and was aimed to study 1) the effect of initial persulfate level of both ferrous and alkaline activated persulfate in pathogen inactivation; 2) the effect of gradual addition of ferrous ion for persulfate activation on pathogen inactivation; 3) the stability of activated persulfate for pathogen inactivation. In addition, the steady-state concentrations of hydroxyl and sulfate radicals were measured. *E. coli* O157:H7 and *Listeria monocytogenes* were selected as the model pathogen for this study because they are among the most common found pathogens in various foodborne outbreaks and they represent different gram stain types with different cell membrane and cell wall structures.

4.2 Materials and methods

4.2.1 Pathogen cultures

Five strains of *E. coli* O157:H7 and *L. monocytogenes* were selected. The strains selected for *E. coli* O157:H7 were 1 (beef isolate), 4 (human isolate), 5 (human isolate), E009 (beef isolate), and 932 (human isolate). The strains selected for *L. monocytogenes* were Scott A (human isolate), LCDC 81-861 (raw cabbage isolate), F8027 (celery isolate), H77-50 (hot dog isolate), and F8369 (corn isolate). All strains were acquired from CDC, USDA or CFS at University of Georgia. All strains were nalidixic acid adapted. Each strain (stored in – 70 ^{o}C) was recovered by sub-culturing in tryptic soy broth (TSB, Difco, Sparks, MD, USA) (*E. coli* O157:H7) or brain heart infusion broth (BHIB, Difco, Sparks, MD, USA) at 37 ^{o}C for 24 h. The resulting culture broth was sub-cultured in nalidixic acid (50 mg/L) supplemented TSB (TSBNA) or BHIB (BHIBNA) at 37 ^{o}C for 24 h. Selective media MacConkey agar (Criterion, Santa Maria, CA, USA) and Palcam Listeria selective agar (Bioworld, Dublin, OH, USA) supplemented with Palcam Listeria selective supplement (EMD Millipore Corporation Billerica, MA, USA) were used to confirm there was no contamination in the resulting culture of *E. coli* O157:H7 and *L. monocytogenes* by direct streaking and incubation at 37 ^{o}C for 24 h and 48 h, respectively. After confirmation, the final resulting cultures were used as stock cultures. Working cultures were prepared by sub-culturing the stock cultures twice in TSBNA (*E. coli* O157:H7) or BHIBNA (*L. monocytogenes*) at 37 ^{o}C for 24 h. The pathogen cells were condensed to cell pellets by centrifuging the sub-cultured broth at 3000×g for 12 min, followed by washing with 20 mmol/L phosphate buffer saline (PBS, pH 7) and re-suspending in PBS. A five-strain cocktail of each pathogen was obtained by mixing equal volume of each five strains.

4.2.2 Treatment with activated persulfate

4.2.2.1 Effect of initial persulfate concentration

The treatment using activated persulfate on each pathogen cocktail was conducted in a 150 mL-sterile glass beaker. Firstly, pre-calculated amount of sterile water and sodium persulfate (Sigma-Aldrich, St. Louis, MO, USA) were added into the beaker. Then 1 mL pathogen cocktail was added into the beaker. The treatment was started by spiking specific amount of 200 mmol/L ferrous sulfate solution or 1 mol/L sodium hydroxide solution (Fisher Scientific, Fair Lawn, NJ, USA) into the beaker. The final volume of the mixture in the beaker after adding all needed chemicals and pathogen cocktails was 25 mL. The beaker was placed on a magnetic plate and a magnetic stir bar was placed inside the beaker to keep the mixture homogenized. The persulfate to activator molar ratios in the mixture were selected based on our previous study (Qi et al., 2018) and were set as follows: persulfate to ferrous at 3:1 for ferrous activation, 20 mmol/L (*E. coli* O157:H7) or 200 mmol/L (*L. monocytogenes*) sodium hydroxide for alkaline activation. One mL was withdrawn from the mixture after 60 and 120 s and neutralized with 9 mL neutralizing buffer (5 g/L sodium thiosulfate with 20 mmol/L PBS for ferrous activation or 100 mmol/L PBS for alkaline activation).

4.2.2.2 Effect of gradual addition of ferrous ion

The treatment setup was similar to section 4.2.2.1 that pre-calculated amount of sodium persulfate and 1 mL pathogen cocktail were mixed together first using sterile water. Then, the treatment was initiated by spiking the ferrous ion gradually (in two or four portions) into the mixture at different time points. For instance, if 30 mmol/L of initial persulfate was used and the ferrous ion was supposed to be spiked in four portions, 0.31 mL of the 200 mmol/L ferrous sulfate solution would be spiked into the mixture at 0, 30, 60, and 90 s. The total amount of

spiked ferrous ion will still be 10 mmol/L in the final mixture. For two portion addition, the ferrous ion will be spiked at 0 and 60 s with 0.625 mL of the ferrous solution being added at each time point. The total reaction time was 2 min.

4.2.2.3 Stability of activated persulfate in pathogen inactivation

The treatment setup was similar as described in section 4.2.2.1. However, the persulfate and the activator solution were mixed first without pathogen spiking. The activated persulfate solution was kept for 3 h and the pathogen cocktail was spiked at 1, 2, and 3 h. At each time point, 1 mL pathogen cocktail was spiked and treated for 2 min before withdrawing 1 mL for microbiological analysis.

4.2.3 Microbiological analysis

Pathogen populations after the treatment were determined by plating 0.1 mL of 10-fold serial diluted neutralized samples onto sodium pyruvate supplemented (0.1%) tryptic soy agar (TSASP for *E. coli* O157:H7) or brain heart infusion agar (BHIASP for *L. monocytogenes*) in duplicate. In addition, 0.25 mL of non-diluted samples were also directly plated in quadruplicate for quantification of samples with low populations. The TSASP plates were incubated at 37 ^{o}C for 24 h while the BHIASP plates were incubated at 37 ^{o}C for 48 h before colony enumeration. For samples that showed no growth by direct plating, enrichment was conducted by transferring 1 mL of the neutralized sample into 9 mL sodium pyruvate supplemented (0.1%) TSB (*E. coli* O157:H7) or BHIB (*L. monocytogenes*). Both broths were incubated at 37 ^{o}C for 24 h and streaked onto respective selective agars. The quantification limit of direct plating was 1.00 log CFU/mL. Samples showing positive enrichment but no growth by direct plating were considered at 0.99 log CFU/mL while samples showing negative enrichment were considered at 0.00 log CFU/mL.

4.2.4 Determination of sulfate and hydroxyl radicals

The steady-state concentrations of sulfate and hydroxyl radicals in activated persulfate solutions were quantified using our previous method (Qi et al., 2018). Briefly, 1 mL of chemical probe solution (2.5 mmol/L anisole and 2.5 mmol/L nitrobenzene) was used to replace the pathogen cocktail and spiked in activated persulfate solutions as described in section 4.2.2. Three milliliters were withdrawn after 120 s for ferrous activation. Three milliliter were withdrawn after 4 and 8 h (*E. coli* O157:H7 conditions) or 1.5 and 6 h (*L. monocytogenes* conditions) for alkaline activation. The withdrawn samples were mixed with 3 mL acetonitrile immediately and extracted with the QuEChERS method (Anastassiades, Lehotay, Stajnbaher, & Schenck, 2003). The final extracted samples were analyzed with a Shimadzu Prominence HPLC System (Shimadzu Scientific Instruments, Columbia, MD, USA).

4.2.5 Statistical analysis

The entire experiment was repeated three times. Excel (Micro software, USA) was used to calculate means, standard deviations, and correlation coefficients. SAS software 9.4 (SAS Institute Inc., Cary, NC, USA) was used to conduct one-way analysis of variance and Duncan's multiple range test using proc glm procedure. Significant difference was considered at $P < 0.05$.

4.3 Results and discussion

4.3.1 Effect of initial persulfate concentration on ferrous activated persulfate

Table 4.1 shows the reductions of *E. coli* O157:H7 and *L. monocytogenes* after ferrous activated persulfate treatment. The initial persulfate concentration was set at 10 to 80 mmol/L with the persulfate to ferrous molar ratio fixed at 3:1 because this ratio can maximize the pathogen inactivation efficacy (Qi et al., 2018). The initial populations were 8.41 ± 0.05 log

CFU/mL (*E. coli* O157:H7) and 8.68 ± 0.05 (*L. monocytogenes*). Persulfate itself at 80 mmol/L did not have any inactivation efficacy on either pathogen (data not shown). For *E. coli* O157:H7, 60 s ferrous activated persulfate treatment achieved 4.18 to 8.41 log CFU/mL reductions depending on the initial persulfate concentrations. Generally, higher initial persulfate concentration lead to significantly higher *E. coli* O157:H7 reductions. For example, by increasing the initial persulfate concentration from 10 to 60 mmol/L, the reduction after 60 s treatment increased from 4.18 to 7.42 log CFU/mL. Further increase of initial persulfate from 60 to 70 and 80 mmol/L lead to 8.41 log CFU/mL reductions, indicating the *E. coli* O157:H7 population was reduced to below the detection limit and no recovery was found after enrichment. Increase of treatment time to 120 s also led to more *E. coli* O157:H7 reductions, ranging from 5.02 to 8.41 log CFU/mL. However, the improvements of reduction from 60 to 120 s were only about 1 log CFU/mL and were not significant ($P \geq 0.05$) at each respective persulfate level. More than 7 log CFU/mL reductions were found with persulfate level at 30 mmol/L or higher while 8.41 log CFU/mL reductions were observed after the initial persulfate was increased to 50 mmol/L.

The same reduction trend was also found for *L. monocytogenes*. During the first 60 s treatment period, 3.51 to 6.63 log CFU/mL reductions were achieved. By increasing the initial persulfate concentration, higher reductions were observed. The increase of initial persulfate level from 10 to 20 mmol/L significantly ($P < 0.05$) increased the reduction from 3.51 to 5.46 log CFU/mL (Table 4.1) and the following increase of persulfate to 40 mmol/L significantly ($P < 0.05$) increased the reduction to 6.26 log CFU/mL. However, no significant increase of reduction was found when the initial persulfate level was increased from 40 to 80 mmol/L (6.26 to 6.63 log CFU/mL reductions). Further increase of treatment time to 120 s led to higher reductions (4.45 to

8.35 log CFU/mL), though the increases were generally less than 1 log CFU/mL. Different to *E. coli* O157:H7, *L. monocytogenes* showed partial positive enrichment results even when the initial persulfate concentration was increased to 80 mmol/L. The maximum reduction of *L. monocytogenes* was 8.35 log CFU/mL. Although higher *L. monocytogenes* reductions were achieved by increasing the persulfate concentration from 40 to 70 mmol/L, the increases were not significant ($P \geq 0.05$) (7.10 to 7.34 log CFU/mL reductions) while significantly ($P < 0.05$) more reduction was only observed with further increase of initial persulfate to 80 mmol/L. This is in accordance with our previous study (Qi et al., 2018) that *L. monocytogenes* was more resistant against activated persulfate treatment than *E. coli* O157:H7. This might be due to the thicker cell wall of Gram-positive bacteria that is less susceptible to AOP-based treatment as demonstrated by many studies (Barnes, Molina, Xu, Thompson, & Dobson, 2013; Kim et al., 2013; Zhang et al., 2018). On the other hand, the microbial reduction trends generally coincided with the hydroxyl and sulfate radical steady-state concentration changes. When the initial persulfate level was increased from 10 to 80 mmol/mL, $[HO\cdot]_{ss}$ increased from 9.14×10^{-14} to 1.41×10^{-13} mol/L and $[SO_4^{-}\cdot]_{ss}$ increased from 4.11×10^{-14} to 1.74×10^{-13} mol/L. The concentrations of free radicals and the pathogen reduction rates are positively correlated. The correlation coefficients are 0.69 and 0.96 for *E. coli* O157:H7 reductions and 0.86 and 0.96 for *L. monocytogenes* reductions against hydroxyl and sulfate radical concentrations, respectively (Table 4.4). As our previous study demonstrated, free radicals are the major contributing compounds of activated persulfate in pathogen inactivation. Hence, it is reasonable to assume the increasing pathogen inactivation efficacy at higher initial persulfate levels are due to the higher free radical concentration being generated.

Similar findings were reported in other literature. Samyoung et al. (2013) investigated the efficacy of iron-activated persulfate in disinfecting ballast water and they found by increasing the initial persulfate level, the inactivation kinetics of both *Dunaliella tertiolecta* (green alga) and *Pseudonitzschia delicatissima* (marine diatom) were dramatically increased. Wordofa et al. (2017) also demonstrated that the inactivation rate of *E. coli* O157:H7 can be significantly increased by increasing the initial persulfate concentration when iron was used as the activator. More similar findings can be found in environmental studies where environmental pollutants are the primary degradation targets. When ferrous was used as the activator, higher initial persulfate levels can result in improved degradation rates of acid orange 7 (Lei, Zhang, Wang, & Ai, 2015), decabromodiphenyl ether (Peng et al., 2017), trichloroethylene (Liang, Bruell, Marley, & Sperry, 2004b), and gasoline pollutants (Liang, Huang, & Chen, 2008). However, most of these studies did not measure the steady-state concentrations of free radicals produced in their ferrous activated persulfate system but only assumed more free radicals were being generated at higher initial persulfate levels.

4.3.2 Effect of initial persulfate concentration on alkaline activated persulfate

Sodium hydroxide was the alkaline reagent used for persulfate alkaline activation. Different activation conditions were used for *E. coli* O157:H7 and *L. monocytogenes* based on our previous findings (Qi et al., 2018). For *E. coli* O157:H7, the sodium hydroxide concentration was set at 20 mmol/L while the initial persulfate level was changed from 10 to 80 mmol/L. Sodium hydroxide was fixed at 20 mmol/L because this level does not have a strong antimicrobial effect (less than 2 log CFU/mL reduction after 120 s) while is enough to activate persulfate for pathogen inactivation (Qi et al., 2018). The results of *E. coli* O157:H7 reduction are shown in Table 4.2. After 60 s treatment, 3.97 to 4.99 log CFU/mL reductions were achieved.

The maximum reduction was found at maximum persulfate level (80 mmol/L). Although more reductions were obtained by increasing the initial persulfate concentration, the increases of reduction were limited (about 1 log CFU/mL) and were mostly not significant ($P \geq 0.05$). Further increase of treatment time to 120 s achieved more reductions (4.75 to 6.73 log CFU/mL). Similarly, higher initial persulfate concentration achieved more reductions while significantly more reduction was not observed until the persulfate level was increased to 50 mmol/L. Also, the increases of reduction from 60 to 120 s were generally low (less than 1 log CFU/mL) when the initial persulfate concentrations were at 10 to 40 mmol/L. However, more than 1.5 log CFU/mL increase of reduction was observed by extending the treatment from 60 to 120 s when the initial persulfate concentrations were at 50 mmol/L or higher. Similar to ferrous activated persulfate, both $[HO\cdot]_{ss}$ (3.42×10^{-16} to 3.23×10^{-15} mol/L) and $[SO_4^{-}\cdot]_s$ (1.17×10^{-16} to 1.52×10^{-14} mol/L) radical concentrations were found positively correlated with initial persulfate levels. The correlation coefficients are 0.95 and 0.96 against hydroxyl and sulfate radicals, respectively (Table 4.4).

For *L. monocytogenes*, a different activation condition was used because *L. monocytogenes* is much more resistant to alkaline activated persulfate (Qi et al., 2018). Sodium hydroxide at 200 mmol/L was used for activation and the initial persulfate concentration was changed from 100 to 800 mmol/L. Sodium hydroxide at 200 mmol/L showed very limited inactivation efficacy against *L. monocytogenes* (less than 0.5 log CFU/mL reduction in 120 s) (Qi et al., 2018) and persulfate alone at 800 mmol/L showed no inactivation efficacy against *L. monocytogenes* (data not shown). After 60 s treatment, 0.65 to 6.44 log CFU/mL reductions were achieved. Different from *E. coli* O157:H7, the increase of initial persulfate concentration dramatically increased the reduction of *L. monocytogenes* (Table 4.3). For example, 100 mmol/L

initial persulfate only obtained 0.65 log CFU/L reduction while the increase of initial persulfate to 400 and 800 achieved 2.59 and 6.44 log CFU/mL reductions, respectively. The extension of treatment to 120 s resulted in significantly more reductions (about 2 to 4 log CFU/mL more reductions) than treatment at 60 s. Also, 8.68 log CFU/mL reduction was found at 600 to 800 mmol/L initial persulfate concentrations. The increasing trend of *L. monocytogenes* reduction was also positively correlated to the free radical steady-state concentrations (r^2 = 0.94 and 0.86 against $[HO\cdot]_{ss}$ and $[SO_4^{-}\cdot]_{ss}$, respectively) (Table 4.4). By increasing the initial persulfate concentration from 100 to 800 mmol/L, $[HO\cdot]_{ss}$ increased from 1.31×10^{-15} to 1.64×10^{-14} mol/L and $[SO^{-}_4\cdot]_{ss}$ increased from 3.84×10^{-15} to 8.21×10^{-15} mol/L.

Studies that focus on the efficacy of alkaline activated persulfate on microbial inactivation are rare. To our best knowledge, our previous study (Qi et al., 2018) was the only one that investigated the potential of alkaline activated persulfate for foodborne pathogen inactivation. However, similar results can be found in environmental treatment with focus on chemical degradation. Lominchar, Santos, de Miguel, and Romero (2018) found elevated initial persulfate concentration using sodium hydroxide activation could achieve higher degradation efficacies on diesel-contaminated soil. Peng et al. (2017) also demonstrated that the increase of initial persulfate dosage from 20 to 500 mmol/L can dramatically increase the degradation kinetic of BDE209 when pH was controlled at 12.20. However, effective chemical degradation by alkaline activated persulfate usually takes more than hours while the current study demonstrated effective pathogen inactivation can be achieved in minutes by alkaline activated persulfate at appropriate initial persulfate concentrations. This short reaction time makes alkaline activated persulfate potentially useful for produce sanitation because fresh produce will usually be washed in the sanitation tank for only a few minutes (Meireles et al., 2016).

4.3.3 Effect of gradual addition of ferrous ion on pathogen inactivation

The optimum persulfate to ferrous ratio (3 : 1) for pathogen inactivation was reported from our previous study (Qi et al., 2018) and the efficacy of ferrous activated persulfate can be improved by increasing the initial persulfate concentration as demonstrated in the current study. However, there are other potential ways to further increase the pathogen inactivation efficacy of ferrous activated persulfate. Ferrous ions can work as the persulfate activator to generate sulfate radicals (Eq. 4.1) while it can also react with sulfate radicals and function as radical scavengers (Eq. 4.5). One way to maximize the activation function and minimize the scavenging function is to spike ferrous ion gradually into persulfate solution instead of adding all ferrous ions at once (Liang, Bruell, Marley, & Sperry, 2004a). In this way, the availability of ferrous ions will be increased and more ferrous ion will react with persulfate to generate free radicals and thus the scavenging effect can be limited.

$$SO_4^{-}\cdot + Fe^{2+} \rightarrow Fe^{3+} + SO_4^{2-} \quad (4.5)$$

Persulfate with initial concentrations of 10, 20, and 30 mmol/L were selected and the corresponding amount of ferrous ions were spiked into the treatment mixture, either at once at the beginning or gradually in two or four portions. Both *E. coli* O157:H7 and *L. monocytogenes* were treated with direct or sequential addition of ferrous for 120 s. Additional reductions were observed for both pathogens when ferrous ions were added gradually (Table 4.5). However, compared to adding ferrous at once, adding ferrous in two portions did not achieve significantly ($P \geq 0.05$) more (less than 0.5 log CFU/mL) reductions (Table 4.5). Adding ferrous ion in four portions showed significantly ($P < 0.05$) more (0.5 to 1.0 log CFU/mL) reductions of both

pathogens than adding ferrous at once, except for *E. coli* O157:H7 treated with 30 mmol/L initial persulfate where the reduction was only improved from 7.15 to 7.39 log CFU/mL. That might be because 7.15 log CFU/mL reduction was already very high and further increase of reduction may need much more free radicals. The steady-state concentrations of both hydroxyl and sulfate radicals were also measured for each condition. Gradual addition of ferrous ion achieved higher steady-state concentrations of hydroxyl radicals, while the concentrations of sulfate radical fluctuated. Since hydroxyl radicals were generated by sulfate radicals and water (Eq. 4.2), the gradual addition of ferrous ions may push more sulfate radicals to react with water molecule and resulted in fluctuation of sulfate radical concentration. More importantly, as hydroxyl radicals were the primary compound for pathogen inactivation (Qi et al., 2018), the increased inactivation efficacy by gradual ferrous addition can be well explained by the increasing amount of hydroxyl radicals.

The improved efficiency of sequential addition of ferrous ion was also demonstrated by Liang et al. (2004a) who found five sequential addition of ferrous ion into persulfate can achieve up to 31% more degradation of trichloroethylene. Instead of sequential addition, this slow spiking of ferrous ion can also be achieved using chelating agents. Chelating agents can trap the ferrous ions in the solution and slowly release them for persulfate activation (Matzek & Carter, 2016). Different chelating agents, such as EDTA, citric acid, and ascorbic acid, have been demonstrated as effective ferrous chelators to improve the degradation efficacy against trichloroethylene (Liang et al., 2004b; Lin, Liang, & Yu, 2016; Wu et al., 2014). The use of chelating agents (EDTA and citric acid) were also tested in the current study (data not shown). However, no improvement on pathogen inactivation was observed. In addition, another way to improve the availability of ferrous ion and free radical concentration is to add reducing reagents

so that the generated ferric ion (Eq. 4.1) can be reduced back to ferrous ion and continue to activate persulfate. Wordofa et al. (2017) used hydroxylamine as the reducing reagent and showed improved inactivation efficacy on *E. coli* O157:H7 in water solution. Hydroxylamine, however, is a relatively toxic chemical and cannot be applied for food samples. Therefore, hydroxylamine was not tested in the current study.

4.3.4 Stability of activated persulfate on pathogen inactivation

One challenge that may limit the application of activated persulfate for produce washing is how long the efficacy can last because the major pathogen inactivation power comes from the persulfate activation reaction. Our previous study (Qi et al., 2018) demonstrated alkaline activation can last for hours and the radical producing power can be maintained. Ferrous activated persulfate, on the other hand, will go through a relatively faster activation in the first few minutes and then changed into a relative slower activation status where only sulfate radicals are being produced. In order to test if such changes during activation have any effect on the pathogen inactivation efficacy, the *E. coli* O157:H7 and *L. monocytogenes* cocktail was spiked into ferrous and alkaline activated persulfate 1 h after activation as described in section 4.2.2.3. Another challenge to apply activated persulfate for produce sanitation is whether they can be repeatedly used by continuously introducing new food samples. Continuously introducing new food samples can build up the overall microbial load in the activated persulfate solution which may scavenge free radicals and limit the overall inactivation efficacy. To investigate such potential effect, the treatment mixture was kept for another one (as 2 h for "Time after activation" on Table 4.6) and two hours (as 3 h for "Time after activation" on Table 4.6) after the initial pathogen spiking (as 1 h for "Time after activation" on Table 4.6) and was spiked with new pathogen cocktails again for each additional hour (section 4.2.2.3).

The results are shown in Table 4.6. For ferrous activation, no significant ($P \geq 0.05$) difference in reduction was found for either pathogen between 0-h and 1-h activation, indicating the pathogen inactivation efficacy of ferrous activated persulfate can be stable for a long period of time. The inactivation efficacy on *E. coli* O157:H7 was still maintained at 7.45 log CFU/mL even after the additional inoculum spiking (2 h) while the reduction of *L. monocytogenes* dropped significantly ($P < 0.05$) from 6.60 (1-h activation) to 4.85 log CFU/mL (2-h activation). The third spiking at 3-hour after activation lead to significant drop of *E. coli* O157:H7 reduction to 6.80 log CFU/mL while the reduction of *L. monocytogenes* did not show additional significant ($P \geq 0.05$) drops. These results indicate the pathogen inactivation efficacy could be significantly reduced by continuous use (through introducing additional pathogens) that may lead to organic load build-up and limit the power of free radicals. This finding is in accordance with the study of Yemmireddy and Hung (2015) who demonstrated that food processing water with organic matter can significantly decrease the efficacy of UV light activated titanium dioxide-based AOP in inactivating *E. coli* O157:H7.

Alkaline activated persulfate, however, showed a different reduction trend. Compared to the initial reductions, the inactivation efficacy of alkaline activated persulfate was increased significantly ($P < 0.05$) to 6.12 and 7.27 log CFU/mL for *E. coli* O157:H7 and *L. monocytogenes*, respectively, after 1-hour activation. This might be because the reaction between persulfate and sodium hydroxide is relatively slow and needs more time to reach equilibrium and maximize the free radical production. The second and third spiking at 2- and 3-hour after activation led to significant decrease of reductions for *E. coli* O157:H7 while the reduction on *L. monocytogenes* did not show significant ($P \geq 0.05$) changes. This is probably because the sodium hydroxide levels used for persulfate activation are different between *E. coli* O157:H7 and *L.*

monocytogenes. During the 3-hour activation period, the pathogen cocktail was spiked three times into the activated persulfate solution. The pathogen cocktail was prepared in 20 mmol/L PBS which has buffering effect and can neutralize a certain amount of sodium hydroxide after each spiking. For *E. coli* O157:H7 that was treated with a lower sodium hydroxide level (20 mmol/L), this neutralizing effect can cause significant pH change and reduce the activating power on persulfate. As a result, fewer free radicals may be produced and therefore lower pathogen reduction efficacy was achieved. *L. monocytogenes*, on the other hand, was treated with a higher sodium hydroxide level (200 mmol/L). The neutralizing effect of PBS may not be significant for such high sodium hydroxide concentration with only three spikes. Hence, the inactivation of *L. monocytogenes* can be maintained. This indicates the pH must be actively monitored for alkaline activation to maintain maximum activation and pathogen inactivation efficacy.

4.4 Conclusions

The efficacy of ferrous and alkaline activated persulfate in inactivation of *E. coli* O157:H7 and *L. monocytogenes* was explored. The results showed the initial persulfate concentration had a significant effect on the overall pathogen inactivation efficacy. Higher initial persulfate levels can produce more free radicals and achieve higher pathogen reductions. The efficacy of ferrous activated persulfate can also be influenced by the ferrous ion availability. Adding ferrous ion into persulfate gradually can significantly improve the pathogen inactivation efficacy compared to adding all ferrous ions at once. Furthermore, the pathogen inactivation efficacy of both ferrous and alkaline activated persulfate can last for a long period of time (more than 1 hour) and the activated persulfate solution can be reused for pathogen inactivation several

times. Overall, the results from the current study are promising because more information is gained on the efficacy of activated persulfate in pathogen inactivation. Based on our data, we found activated persulfate solution can be very effective for fresh produce washing applications.

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Table 4.1. Efficacy of ferrous activated persulfate in inactivating *E. coli* O157:H7 and *L. monocytogenes* at different initial persulfate levels and the measured hydroxyl and sulfate radical concentrations.

Initial PS levels (mmol/L)	Reduction (log CFU/mL)				$[HO\cdot]_{ss}$ (mol/L)	$[SO_4^{-}\cdot]_{ss}$ (mol/L)
	E. coli O157:H7		*L. monocytogenes*			
	(60 s)	(120 s)	(60 s)	(120 s)		
10	4.18 ± 0.69 E	5.02 ± 0.19 E	3.51 ± 0.13 C	4.45 ± 0.09 E	9.14×10^{-14}	4.11×10^{-14}
20	5.75 ± 0.17 D	6.43 ± 0.25 D	5.46 ± 0.09 B	5.59 ± 0.15 D	9.48×10^{-14}	6.28×10^{-14}
30	6.52 ± 0.55 C	7.15 ± 0.45 C	5.61 ± 0.45 B	6.38 ± 0.72 CD	9.82×10^{-14}	9.52×10^{-14}
40	6.39 ± 0.26 C	7.75 ± 0.57 B	6.26 ± 0.10 A	7.10 ± 0.11 BC	1.04×10^{-13}	1.25×10^{-13}
50	6.71 ± 0.46 C	8.41 ± 0.00 A	6.57 ± 0.24 A	6.87 ± 0.53 BC	1.01×10^{-13}	1.48×10^{-13}
60	7.42 ± 0.00 B	8.41 ± 0.00 A	6.47 ± 0.38 A	7.48 ± 0.17 B	1.15×10^{-13}	1.45×10^{-13}
70	8.41 ± 0.00 A	8.41 ± 0.00 A	6.76 ± 0.54 A	7.34 ± 0.61 B	1.21×10^{-13}	1.55×10^{-13}
80	8.41 ± 0.00 A	8.41 ± 0.00 A	6.63 ± 0.25 A	8.35 ± 0.57 A	1.41×10^{-13}	1.74×10^{-13}

The initial populations were 8.41 ± 0.05 log CFU/mL (*E. coli* O157:H7) and 8.68 ± 0.05 (*L. monocytogenes*). The persulfate to ferrous molar ratio was set at 3:1 in all conditions. Means (n = 3) in the same column followed by the same capital letters are not significantly different ($P \geq 0.05$). PS = persulfate. $[\ ]_{ss}$ indicates the steady-state concentration of the free radical.

Table 4.2. Efficacy of sodium hydroxide activated persulfate in inactivating *E. coli* O157:H7 at different initial persulfate levels and the measured hydroxyl and sulfate radical concentrations.

Initial PS levels (mmol/L)	Reduction (log CFU/mL)		$[HO\cdot]_{ss}$ (mol/L)	$[SO^{-}_{4}\cdot]_{ss}$ (mol/L)
	(60 s)	(120 s)		
10	3.97 ± 0.20 D	4.81 ± 0.62 D	3.42×10^{-16}	1.17×10^{-16}
20	4.52 ± 0.28 BC	5.06 ± 0.45 D	4.56×10^{-16}	5.25×10^{-16}
30	4.15 ± 0.30 CD	4.75 ± 0.06 D	6.67×10^{-16}	5.23×10^{-16}
40	4.47 ± 0.29 BC	5.33 ± 0.15 CD	1.04×10^{-15}	1.12×10^{-15}
50	4.33 ± 0.23 BCD	5.88 ± 0.60 BC	1.21×10^{-15}	4.33×10^{-15}
60	4.41 ± 0.26 BCD	5.95 ± 0.50 ABC	2.09×10^{-15}	8.87×10^{-15}
70	4.79 ± 0.20 AB	6.56 ± 0.20 AB	2.32×10^{-15}	1.20×10^{-14}
80	4.99 ± 0.26 A	6.73 ± 0.60 A	3.23×10^{-15}	1.52×10^{-14}

The initial *E. coli* O157:H7 population was 8.41 ± 0.04. Sodium hydroxide was set at 20 mmol/L at all treatment conditions. Means (n = 3) in the same column followed by the same capital letters are not significantly different ($P \geq 0.05$). PS = persulfate. $[\ ]_{ss}$ indicates the steady-state concentration of the free radical.

Table 4.3. Efficacy of sodium hydroxide activated persulfate in inactivating *L. monocytogenes* at different initial persulfate concentrations and the measured hydroxyl and sulfate radical concentrations.

Initial PS levels (mmol/L)	Reduction (log CFU/mL)		$[HO\cdot]_{ss}$ (mol/L)	$[SO_4^{-}\cdot]_{ss}$ (mol/L)
	(60 s)	(120 s)		
100	0.65 ± 0.10 F	1.68 ± 0.00 F	1.31×10^{-15}	3.84×10^{-15}
200	1.33 ± 0.14 E	3.48 ± 0.41 E	2.08×10^{-15}	3.55×10^{-15}
300	2.14 ± 0.11 D	4.76 ± 0.43 D	4.06×10^{-15}	4.24×10^{-15}
400	2.59 ± 0.36 D	6.15 ± 0.28 C	6.60×10^{-15}	3.50×10^{-15}
500	3.40 ± 0.15 C	7.58 ± 0.18 B	9.34×10^{-15}	7.10×10^{-15}
600	5.87 ± 0.20 B	8.68 ± 0.00 A	1.18×10^{-14}	6.99×10^{-15}
700	5.77 ± 0.74 B	8.68 ± 0.00 A	1.44×10^{-14}	7.61×10^{-15}
800	6.44 ± 0.28 A	8.68 ± 0.00 A	1.64×10^{-14}	8.21×10^{-15}

The initial *L. monocytogenes* population was 8.68 ± 0.04. Sodium hydroxide was set at 200 mM at all treatment conditions. Means (n = 3) in the same column followed by the same capital letters are not significantly different ($P \geq 0.05$). PS = persulfate. $[\ ]_{ss}$ indicates the steady-state concentration of the free radical.

Table 4.4. Correlation coefficients between pathogen reductions and the steady-state concentration of free radicals.

r^2	Ferrous activation		Alkaline activation	
	$[HO\cdot]_{ss}$	$[SO_4^{-}\cdot]_{ss}$	$[HO\cdot]_{ss}$	$[SO_4^{-}\cdot]_{ss}$
E. coli O157:H7	0.69	0.96	0.95	0.96
L. monocytogenes	0.86	0.96	0.94	0.86

Table 4.5. Effect of sequential addition of ferrous ion on inactivation of *E. coli* O157:H7 and *L. monocytogenes* and the measured hydroxyl and sulfate radical concentrations.

Initial PS levels and number of ferrous additions	Reduction (log CFU/mL)		$[HO\cdot]_{ss}$ (mol/L)	$[SO_4^{-}\cdot]_{ss}$ (mol/L)
	E. coli O157:H7 (120 s)	*L. monocytogenes* (120 s)		
10 mmol/L + one ferrous addition	5.02 ± 0.19 E	4.45 ± 0.09 F	9.14×10^{-14}	4.11×10^{-14}
10 mmol/L + two ferrous additions	5.13 ± 0.49 E	4.81 ± 0.59 EF	6.47×10^{-14}	1.16×10^{-13}
10 mmol/L + four ferrous additions	5.62 ± 0.22 D	6.15 ± 0.79 CD	1.20×10^{-13}	7.40×10^{-14}
20 mmol/L + one ferrous addition	6.43 ± 0.25 C	5.59 ± 0.15 D	9.48×10^{-14}	6.28×10^{-14}
20 mmol/L + two ferrous additions	6.69 ± 0.27 BC	5.56 ± 0.35 DE	1.21×10^{-13}	3.82×10^{-14}
20 mmol/L + four ferrous additions	7.38 ± 0.01 A	6.98 ± 0.70 ABC	1.89×10^{-13}	6.86×10^{-14}
30 mmol/L + one ferrous addition	7.15 ± 0.45 AB	6.38 ± 0.72 BCD	9.82×10^{-14}	9.52×10^{-14}
30 mmol/L + two ferrous additions	6.60 ± 0.18 BC	7.16 ± 0.43 AB	1.46×10^{-13}	4.32×10^{-14}
30 mmol/L + four ferrous additions	7.39 ± 0.01 A	7.35 ± 0.52 A	1.95×10^{-13}	1.10×10^{-13}

The initial populations were 8.38 ± 0.07 log CFU/mL (*E. coli* O157:H7) and 8.65 ± 0.12 (*L. monocytogenes*). One ferrous addition means the total amount of ferrous was added at the beginning. Two ferrous additions means the total amount of ferrous was equally divided into two portions and were spiked at 0 and 60 s, sequentially. Four ferrous addition means the total amount of ferrous was equally divided into four portions and were spiked at 0, 30, 60, and 90 s, sequentially. The molar ratio of initial persulfate to total ferrous ion added was set at 3:1 in all conditions. Means (n = 3) in the same column followed by the same capital letters are not significantly different ($P \geq 0.05$). PS = persulfate.

Table 4.6. Efficacy of ferrous and alkaline activated persulfate in inactivating *E. coli* O157:H7 and *L. monocytogenes* after long period of activation.

Treatment	Time after activation	Reduction (log CFU/mL)	
		E. coli O157:H7	*L. monocytogenes*
Ferrous activation	0 h*	7.75 ± 0.57 A	7.10 ± 0.11 A
	1 h	7.45 ± 0.00 A	6.60 ± 0.50 A
	2 h	7.45 ± 0.00 A	4.85 ± 0.28 B
	3 h	6.80 ± 0.18 B	5.14 ± 0.46 B
Alkaline activation	0 h*	5.33 ± 0.15 B	6.15 ± 0.28 B
	1 h	6.12 ± 0.22 A	7.27 ± 0.35 A
	2 h	5.09 ± 0.06 B	6.99 ± 0.35 A
	3 h	0.90 ± 0.23 C	7.28 ± 0.35 A

The initial populations were 8.44 ± 0.05 log CFU/mL (*E. coli* O157:H7) and 8.67 ± 0.07 (*L. monocytogenes*). For ferrous activation, 40 mmol/L persulfate and 13.3 mmol/L ferrous sulfate was used for both pathogens. For alkaline activation, 40 mmol/L persulfate and 20 mmol/L sodium hydroxide was used for *E. coli* O157:H7 and 400 mmol/L persulfate and 200 mmol/L sodium hydroxide was used for *L. monocytogenes*. Each pathogen was treated for 2 min. Means (n = 3) in the same column under the same activation treatment followed by the same capital letters are not significantly different ($P \geq 0.05$).

* indicates the values from these rows are from Tables 4.1-3.

CHAPTER 5

INACTIVATION MECHANISM OF FERROUS AND ALKALINE ACTIVATED PERSULFATE ON *ESCHERICHIA COLI* O157:H7 AND *LISTERIA MONOCYTOGENES*

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Abstract

This study aimed to elucidate the mechanism of ferrous and alkaline activated persulfate in inactivating *Escherichia coli* O157:H7 and *Listeria monocytogenes*. A five-strain cocktail of each pathogen was treated with activated persulfate for 5 min. The treated pathogens were plated on both selective and non-selective agars. DNA and protein leakage, TTC-dehydrogenase relative activity, and cellular ultrastructure were analyzed. The results showed there were significant ($P < 0.05$) differences in colony counts between selective and non-selective agars for both pathogens after the activated persulfate treatment, indicating the presence of injured cells. The treated pathogens also showed significant leakages of intracellular DNA and protein content, indicating the cell envelope structures were damaged. The TTC-dehydrogenase relative activity of both pathogens was reduced to around 1% after 5 min treatment, indicating the key enzymes involved in cell metabolism were inhibited. Scanning and transmission electron microscopy imaging demonstrated both persulfate activation treatments caused significant damage on the cell walls and cell membranes. Cytoplasm agglutination and leakages were also observed. Based on the results, we proposed the inactivation mechanisms of ferrous and alkaline activated persulfate on pathogen cells are through breaking cell protective barriers, inhibiting key enzymes activities, and causing leakage of intracellular materials.

Keywords

Advanced oxidation process; Free radicals; Foodborne pathogens; Inactivation mechanism; Electron microscopy

5.1 Introduction

The fresh produce industry has become one of the major components of the global food market due to the nutritional values and benefits to human health (Joshi, Mahendran, Alagusundaram, Norton, & Tiwari, 2013; Feng, et al., 2018). However, fresh produce-related foodborne outbreaks and illnesses have also been increasing (Gil, Selma, López-Gálvez, & Allende, 2009). For instance, leafy vegetables are estimated to be the major cause of foodborne illnesses in the U.S. (Painter, et al., 2013). The contamination of fresh produce can be from both pre-harvest and post-harvest phases (Yeni, Yavas, Alpas, & Soyer, 2016). Once contaminated, consumption of fresh produce can be of great concern as it is usually consumed raw. In the U.S. produce industry, chlorine-based sanitizers are often applied for produce washing to inactivate and remove potential pathogens (Feliziani, Lichter, Smilanick, & Ippolito, 2016). However, chlorine can react with organic matter from produce and generate chlorinated disinfection by-products (DBPs), some of which are potential health threats to consumers (Legay, Rodriguez, Serodes, & Levallois, 2010).

Activated persulfate, a novel advanced oxidation process, has been considered as an alternative sanitizer for fresh produce sanitation. Persulfate ($S_2O_8^{2-}$) can be activated by different activators to generate highly reactive free radicals, such as hydroxyl radicals (Matzek & Carter, 2016). The free radicals have high oxidation-reduction potential and have been demonstrated to be effective in degrading many environmental pollutants (Tsitonaki et al., 2010). Due to the high oxidative power, the free radicals can also be effective in inactivating pathogen cells. For example, hydroxyl radicals generated by hydrogen peroxide photolysis were shown to be effective in killing *Streptococcus mutans* in both planktonic and biofilm form (Nakamura et al., 2016). Our previous study also demonstrated both ferrous and alkaline activated persulfate can

achieve more than 7 log CFU/mL reductions on *E. coli* O157:H7 and *L. monocytogenes* (Qi et al., 2018).

Ferrous sulfate and alkaline reagents are the two common activators to activate persulfate (Matzek & Carter, 2016). Hydroxyl radicals, sulfate radicals or superoxide radicals can be produced from ferrous or alkaline activated persulfate (Eq. 5.1-4). Different free radicals contribute differently in each activation method for pathogen inactivation. As our previous study indicated, hydroxyl radical was the major contributing radical in ferrous activated persulfate to inactivate *E. coli* O157:H7 and *L. monocytogenes* while superoxide radical was the major radical of alkaline activated persulfate in pathogen inactivation (Qi, Huang, & Hung, 2018). Although the major radical species of activated persulfate in pathogen inactivation was deduced, the mechanism of exactly how pathogen cells were inactivated by activated persulfate was still unclear. Both Liang, Shan, Zhang, & Tong (2015) and Zhang, Zou, Cai, Li, & He (2018) indicated the primary disinfection mechanism of reactive oxygen species in their photocatalytic system were through cell membrane damage. However, Zhang, Wu, Zhang, & Yang (2011) indicated the main mechanism of ozonated water in inactivating *Pseudomonas aeruginosa* was by increasing the cytoplasmic membrane permeability and causing cytoplasm coagulation rather than disrupting cell membranes. To our best knowledge, no studies have been conducted to investigate the mechanism of activated persulfate treatment in pathogen inactivation.

$$S_2O_8^{2-} + Fe^{2+} \rightarrow SO_4^{-}\cdot + SO_4^{2-} + Fe^{3+} \quad (5.1)$$

$$SO_4^{-}\cdot + H_2O \rightarrow SO_4^{2-} + OH\cdot + H^{+} \quad (5.2)$$

$$S_2O_8^{2-} + 2H_2O \xrightarrow{OH^-} SO_4^{2-} + SO_4^{-}\cdot + O_2^{-}\cdot + 4H^{+} \quad (5.3)$$

$$SO_4^{-}\cdot + OH^{-} \rightarrow SO_4^{2-} + OH\cdot \quad (5.4)$$

This study was undertaken to investigate the inactivation mechanisms of ferrous and alkaline activated persulfate on *E. coli* O157:H7 and *L. monocytogenes*. Both selective and non-selective agars were used to detect the presence of injured cells after the treatment. The leakages of intracellular DNA and protein were measured. The change of TTC-dehydrogenase relative activity was also recorded. In addition, electron microscopy was used to observe and compare the ultrastructure of both pathogens before and after the treatment.

5.2 Materials and Methods

5.2.1 Chemicals

Sodium persulfate, glucose, and protein standard were from Sigma-Aldrich (St. Louis, MO, USA). Ferrous sulfate, sodium hydroxide solution (10 mol/L), Tris-HCl, 2,3,5-triphenyl tetrazolium chloride (TTC), Bradford dye reagent, and isopropanol alcohol (IPA) were from Fisher Scientific (Fair Lawn, NJ, USA). Sodium persulfate working solution (200 mmol/L), ferrous sulfate working solution (200 mmol/L), and sodium hydroxide working solution (1 mol/L) were freshly prepared in deionized water before each experiment started.

5.2.2 Bacterial cultures

Five nalidixic acid-adapted strains of *E. coli* O157:H7 and *L. monocytogenes* were used. The strains were 1 (beef isolate), 4 (human isolate), 5 (human isolate), E009 (beef isolate), and 932 (human isolate) for *E. coli* O157:H7, and Scott A (human isolate), LCDC 81-861 (raw cabbage isolate), F8027 (celery isolate), H77-50 (hot dog isolate), and F8369 (corn isolate) for *L. monocytogenes*. The five-strain cocktail of each pathogen was prepared using the same method as described in our previous study (Qi et al., 2018).

5.2.3 Treatment

The treatment was conducted in 150 mL sterile glass beakers. Five milliliters of the bacterial cocktail were added to the beaker containing sterile water and 5 mL persulfate working solution or 0.6 g sodium persulfate. To initiate the persulfate activation, 1.7 mL ferrous working solution and 0.5 or 5 mL sodium hydroxide working solution were spiked into the mixture. The total volume of the mixture was at 25 mL. The mixture was kept homogenized with a sterile magnetic bar spinning at high speed on a magnetic stirrer (Hanna Instruments, Woonsocket, RI, USA). One milliliter from the mixture was withdrawn at 1, 2, 3, 4, and 5 min and transferred to 5 mL of neutralizing buffer containing 10 g/L sodium thiosulfate and 100 mmol/L PBS for DNA leakage, TTC-dehydrogenase and electron microscopy analysis or to 1 mol/L IPA and 100 mmol/L PBS for protein leakage analysis. Sodium thiosulfate was not used to neutralize samples for protein leakage analysis because we found sodium thiosulfate can react with the Bradford dye reagent and interfere the color development. IPA was used as an effective radical inhibitor to quench the reaction instead of neutralizing buffer (Qi et al., 2018). Control samples were prepared by replacing the persulfate treatment solution with sterile water.

5.2.4 Measurement of intracellular DNA leakage

The method was based on the work of Zeng et al. (2010). Briefly, 6 mL of the neutralized sample described in section 5.2.3 was centrifuged at 14,000 × g for 3 min. Two milliliters of the supernatant were transferred into a 1 cm quartz cuvette and the absorbance was measured at 260 nm using an Orion AquaMate 8000 UV/Vis spectrophotometer (Thermo Scientific, Waltham, MA, USA). One OD unit was considered equivalent to 50 µg/mL DNA concentration.

5.2.5 Measurement of intracellular protein leakage

The method was based on the work of Zeng et al. (2010). The neutralized sample using IPA described in section 5.2.3 was centrifuged at 14,000 × g for 3 min. One milliliter of the supernatant was withdrawn and stained with 1 mL of Bradford dye reagent for 5 min for color development. Quantification was based on a 5-point external calibration curve prepared with standard protein solutions. The absorbance was measured at 595 nm using the same spectrophotometer described in section 5.2.4.

5.2.6 Measurement of TTC-dehydrogenase relative activity

The measurement was based on the work of Ding et al. (2016) and Burdock, Brooks, & Ghaly (2011). Briefly, 2 mL of Tris-HCl buffer (1.5 mol/L and pH 8.2) and 2 mL TTC-glucose solution (0.4% TTC solution and 0.1 mol/L glucose) were added to 6 mL of neutralized sample described in section 5.2.3. The mixture was incubated at 37 ^{o}C for 2 h to allow the red color development. Then, the 10 mL mixture was centrifuged at 14,000 × g for 3 min. The supernatant was decanted and 5 mL ethanol was added. The mixture was allowed to sit for 30 min with occasional vortex to break cell walls and cell membranes to leach the developed color compound, 2,3,5-triphenylformazan (Ding et al., 2016). After ethanol extraction, the sample was centrifuged at 14,000 × g for 3 min and 2 mL of the supernatant was taken for absorbance measurement at 485 nm. The dehydrogenase relative activity (DRA) was calculated as DRA (%) = $(A_x / A_0) \times 100$, where A_x and A_0 represent the absorbance of treated and control samples, respectively.

5.2.7 Ultrastructure observation by electron microscopy

The neutralized sample described in section 5.2.3 was centrifuged at 3,000 × g for 12 min and the supernatant was removed. The remaining cell precipitate was fixed with 2%

glutaraldehyde (prepared in PBS) for 1 h at 4 ^{o}C. Then, the sample was washed three times with PBS to remove excess glutaraldehyde followed by a secondary fixation in 1% OsO_4/PBS for 1 h at 4 ^{o}C. Afterwards, the sample was washed once in PBS for 10 min and then washed twice in water to remove excess salt followed by dehydration in ethanol series (25% increments of ethanol at 10 min for each step up to 100% ethanol). For SEM, the dehydrated sample was placed on a holder in a critical point dryer (Tousimis 780-A CPD, Rockville, MD). The dried sample was carefully attached to an aluminum SEM stub and coated with gold (Leica EM ACE600, Leica Microsystems GmbH, Vienna, Austria) before observation with a FEI Teneo SEM (FEI Co., Hillsboro, OR, USA). For TEM, the ethanol-dehydrated sample was transferred into 50% propylene oxide (PO) in ethanol for 5 min and then 100% PO with two changes to ensure complete transition to PO. The sample was then infiltrated with EmBed 812 resin (EMS, Harrisburg, PA, USA) in 25% increments of 25% resin/75% PO, 50% resin/50% PO, 75% resin/25% PO, and 100% resin with 1 h between steps. The final sample was infiltrated with 100% EmBed812 again overnight. Then, the sample was placed in a fresh 100% resin and placed in polypropylene molds. The sample was polymerized at 60 ^{o}C overnight. Hardened blocks were trimmed and sectioned on a RMC ultramicrotome (MTX, Boeckeler, Tucson, AZ, USA) to a thickness of approximately 50 nm and collected on a copper grid. Sections were then post-stained with uranyl acetate (EMS, Harrisburg PA, USA) for 30 min and lead citrate (EMS, Harrisburg PA, USA) for 5 min. The prepared specimens were observed with a JEOL JEM1011 TEM (JEOL, Inc., Peabody, MA, USA).

5.2.8 Microbiological analysis

The neutralized samples described in section 5.2.3 were serially diluted in 20 mmol/L PBS and 0.1 mL was plated in duplicate on tryptic soy agar supplemented with 0.1% sodium

pyruvate (TSASP) and sorbitol MacConkey agar (SMAC) for *E. coli* O157:H7 or brain heart infusion agar supplemented with 0.1% sodium pyruvate (BHIASP) and Listeria selective agar supplemented with listeria selective supplement (LSA) for *L. monocytogenes*. To quantify samples with low populations, 0.25 mL non-diluted samples were plated in quadruplicate. Plates for *E. coli* O157:H7 were incubated at 37 ^{o}C for 24 h while plates for *L. monocytogenes* were incubated at 37 ^{o}C for 48 h before colony enumeration.

5.2.9 Statistical analysis

The entire experiment was replicated three times. Means and standard deviations were calculated in Excel (Micro software, USA). SAS software 9.4 (SAS Institute Inc., Cary, NC, USA) was used for statistical analysis. One-way analysis of variance was conducted followed by Duncan's multiple range test with proc glm procedure. A $P < 0.05$ was considered to be significantly different.

5.3 Results and discussion

5.3.1 Survivals on selective and non-selective agar plates

Table 5.1 shows the survival of *E. coli* O157:H7 and *L. monocytogenes* after the activated persulfate treatment. The treatment conditions were selected according to our previous study (Qi et al., 2018). For *E. coli* O157:H7, survival was significantly reduced with the increase of treatment time. As the TSASP (non-selective agar) results show, ferrous activated persulfate treatment significantly ($P < 0.05$) reduced the initial *E. coli* O157:H7 population from 9.28 to 6.44 log CFU/mL after 1 min treatment and the remaining population gradually reduced with the further increase of treatment time. No *E. coli* O157:H7 colony was found on TSASP plates after 5 min treatment. Alkaline activated persulfate achieved a similar reduction trend on *E. coli*

O157:H7. As the TSASP results show, the *E. coli* O157:H7 population was reduced from 9.35 to 1.45 log CFU/mL after 5 min treatment. For both activation methods, no *E. coli* O157:H7 were recovered from the SMAC plates (selective agar) even after only 1 min treatment. The significant differences of colony counts between selective and non-selective agars indicated the *E. coli* O157:H7 cells were injured after the activated persulfate treatment. Injured bacterial cells are able to repair the injury in a favorable environment (Kang & Fung, 1999). However, injured cells are not able to form colonies on selective media because the selective chemicals are targeted for healthy microorganisms and can block the repair mechanism (Back, Kim, Park, Chung, & Kang, 2012). Izumi, Nakata, & Inoue (2016) demonstrated agricultural water added with electrolyzed water can cause 75% injury rate in coliforms and the high oxidation power of chlorine was deemed as the primary injury cause. Because the free radicals produced by activated persulfate were the major compounds in pathogen inactivation (Qi et al., 2018) and they are also highly oxidative, it is reasonable to assume the injury was caused by the free radical attack on pathogen cells.

The plate counts of *L. monocytogenes* were also significantly different between selective and non-selective agars (Table 5.1). The BHIASP (non-selective) results show ferrous activated persulfate reduced the *L. monocytogenes* population from 9.59 to 1.67 log CFU/mL in 5 min while alkaline activated persulfate decreased the *L. monocytogenes* population from 9.48 to 1.75 log CFU/mL. The *L. monocytogenes* colony counts on LSA plates were significantly ($P < 0.05$) lower than that on BHIASP plates at each treatment time level for ferrous activated persulfate treatment. For alkaline activation treatment, LSA plates also always showed lower colony counts than BHIASP plates though not all the differences were significant ($P < 0.05$). These indicated *L. monocytogenes* cells were also injured after the ferrous and alkaline activated persulfate

treatment. However, the recovery rate difference of *L. monocytogenes* between selective media (LSA) and non-selective media (BHIASP) by alkaline activated persulfate were much lower than *E. coli* O157:H7 (Table 5.1). This might be because of the thicker cell wall of gram-positive bacteria that are more resistant against free radical attack (Bogdan, 2015).

5.3.2 Leakage of intracellular DNA and proteins

The results of DNA leakage are shown in Table 5.2. For *E. coli* O157:H7, the DNA leakage increased from 2.00 to 4.37 µg/mL after 1 min ferrous activated persulfate treatment while the DNA leakage increased from 2.08 to 29.00 µg/mL after 1 min alkaline activated persulfate treatment. For both activation treatments, no significant further increase of DNA leakage was observed with further increase of treatment time to 5 min (Table 5.2). For *L. monocytogenes*, the DNA leakage was increased from around 1.90 to 2.58 and 3.92 µg/mL after 1 min ferrous and alkaline activated persulfate treatment, respectively. Similarly, no significant further increase of DNA leakage was observed after 1 min treatment. On the other hand, protein leakage was only measured for alkaline activation treated pathogens. *E. coli* O157:H7 showed a sharp increase in protein leakage from 0.63 to 7.33 µg/mL after 1 min treatment followed by a gradual increase to 12.07 µg/mL in 4 min (Table 5.3). The protein leakage of *L. monocytogenes* increased from 0.53 to around 2.65 µg/mL after 1 min and remained about the same afterwards.

Protein leakage was not reported for ferrous activation treated pathogens because we were not able to observe any protein leakages. It was suspected that the leaked protein quickly reacted with the free radicals (Gatasheh, Subbaram, Kannan, & Naseem, 2017). Therefore, protein standards were used to spike into ferrous and alkaline activated persulfate solutions to observe protein degradation. As Table 5.4 shows, about 50% of protein standards were degraded by ferrous activated persulfate while no degradation was observed by alkaline activated

persulfate. Our previous study showed ferrous activated persulfate can generate about 100 to 1000 times more hydroxyl radicals than alkaline activated persulfate (Qi et al., 2018). Therefore, the reason why no protein leakage was observed by ferrous activation treated pathogens is possibly that the leaked proteins were fully consumed by the hydroxyl radicals at high concentrations. Alkaline activation, on the other hand, generates much fewer hydroxyl radicals that may not be able to cause significant protein degradation. In addition, *E. coli* O157:H7 treated by alkaline activated persulfate showed higher DNA and protein leakages than that of *L. monocytogenes*. This might be because we used higher persulfate and alkaline concentrations (100 mmol/L persulfate and 200 mmol/L NaOH) to treat *L. monocytogenes* that will generate about 10 times more hydroxyl radials compared to the alkaline activation conditions (40 mmol/L persulfate and 20 mmol/L NaOH) used for *E. coli* O157:H7 (Qi et al., 2018).

The measurement of intracellular material leakage has been used by many studies as indicators of bacterial cell envelope damage (Zeng et al., 2010; Liang et al., 2015; Zhang et al., 2018). Since we observed significant leakages of DNA and protein on both pathogens after the activated persulfate treatment, it is highly possible that one of the major inactivation mechanisms is by disrupting cell protective layers.

5.3.3 Measurement of TTC-dehydrogenase relative activity

Table 5.5 shows the dehydrogenase relative activity (DRA) of both pathogens after the activated persulfate treatment. All the treatments showed the DRA dropped from 100% to near 1% within 5 min. For *E. coli* O157:H7, the ferrous activated persulfate treatment resulted in a gradual decrease of DRA from 100% to 0.90% in 5 min. However, the alkaline activated persulfate treatment resulted in a quick drop of DRA from 100% to 2.36% in only 1 min. For *L. monocytogenes*, ferrous activated persulfate treatment also lead to a quick drop of DRA to 2.77%

in the first minute. Alkaline activated persulfate achieved a DRA drop to 23.14% after 1 min and then dropped to 2.75% after 2 min.

Although the major contributing radical in pathogen inactivation are different between ferrous and alkaline activated persulfate (Qi et al., 2018), Table 5.5 demonstrates both activation methods can induce significant inhibition in dehydrogenase activity. Dehydrogenase is a key enzyme in bacterial respiratory chains (Berrisford, Baradaran, & Sazanov, 2016). The significant decrease of DRA indicates the respiration of both pathogens were decreased and anabolism may have developed. Ding et al. (2016) observed significant dehydrogenase activity decrease in *S. aureus* treated with slightly acidic electrolyzed water. They indicated highly oxidative chemicals, such as chlorine in electrolyzed water, could form covalent bond with enzymes to alter their structures. The highly oxidative free radicals generated by ferrous and alkaline activated persulfate might have the same effect as chlorine in denaturing enzyme activities. Thus, another possible inactivation mechanism of ferrous and alkaline activated persulfate on pathogen cells is through the breakdown of key enzymes involved in cellular metabolism.

5.3.4 Ultrastructure of *E. coli* O157:H7 and *L. monocytogenes*

SEM images of *E. coli* O157:H7 are shown in Fig. 5.1. For ferrous activation, 1 min treatment did not result in significant impact on the cell morphology (Fig. 5.1B). The cell surface and cell shape remained intact and showed no significant difference compared to the control cell (Fig. 5.1A). Significant impact was found after 5 min treatment where the cell markedly shrank and deformed with holes formed on the surface (Fig. 5.1C). On the other hand, alkaline activated persulfate resulted in slight damage on *E. coli* O157:H7 surface ultrastructure (Fig. 5.1D) after 1 min treatment. After 5 min treatment, both holes and vesicles appeared on the cell surface, indicating damage and increase of cytoplasm membrane permeability (Fig. 5.1E). On the other

hand, for *L. monocytogenes*, no significant change of cell surface structure and cell shape was found after 1 min ferrous activation treatment (Fig. 5.2B) compared to the control cell (Fig. 5.2A) while significant damage was found after 5 min treatment (Fig. 5.2C). As Fig. 5.2C shows, collapse was found on the top end of the cell and a significant cut was found in the cell body. For alkaline activation, *L. monocytogenes* surface showed slight deformation and shrinkage after 1 min treatment (Fig. 5.2D). Holes and significant shrinkage were found after 5 min treatment, indicating the start of cell lysis (Fig. 5.2E). In addition, for all the SEM images of ferrous activation treated samples, vesicle-like particles can be found in the background or on the cell surfaces. Those are ferric precipitate produced during ferrous activation that could not be removed without affecting SEM observations. Overall, the SEM pictures indicate both ferrous and alkaline activated persulfate treatment can result in significant cell surface damage on pathogen cells, which can lead to cell injury and death. Also, less severe surface damage was found after 1 min of both activation treatment, which is in accordance with the data in Table 5.1 that majority of the pathogen cells were still alive after 1 min activated persulfate treatment.

TEM imaging was only conducted for alkaline activated persulfate treated cells because resin polarization cannot be conducted with the presence of ferric ion generated by ferrous activated persulfate. Fig. 5.3 shows the cytoplasm ultrastructure of *E. coli* O157:H7. The control cell (Fig. 5.3A) is spherical with intact cell envelope and evenly distributed cytoplasm. The nucleoid was also intact. After 1 min treatment, the cell shape was still maintained but the cell wall and cell membrane became obscure (Fig. 5.3B). Also, the cytoplasm started to condense and agglutinate, which might be due to the formation of vacuoles and the leakage of cytoplasm material. After 5 min treatment, significant cell wall degradation was observed and the cytoplasm color was much lighter, indicating the significant leakage of intracellular material

(Fig. 5.3C). The TEM images of *L. monocytogenes* are shown in Fig. 5.4. The untreated cell (Fig. 5.4A) was elliptical in shape with a thicker cell wall compared to *E. coli* O157:H7 (Fig. 5.3A). The cytoplasm was also evenly distributed with nucleoid located in the center. Similar to *E. coli* O157:H7, after 1 min treatment, *L. monocytogenes* developed agglutination in cytoplasm while no significant change of cell shape was observed (Fig. 5.4B). Significant damage was found on cell walls (Fig. 5.4C) after 5 min. Part of the cell wall was cleaved and the cytoplasm became much less dense. Overall, the TEM pictures confirmed the SEM results that 1 min of ferrous and alkaline activation treatment would not cause significant damage on cell envelope integrities while 5 min treatment can cause fatal effects on both outer and inner cell structures. In addition, the agglutination and fading of cytoplasm after activated persulfate treatment further confirms the leakage of intracellular material as indicated by the data in Tables 5.3-4.

Other researchers also found similar effects on the pathogen cell ultrastructure using advanced oxidation process or highly oxidative chemical treatments. Kim et al. (2013) applied ultraviolet-assisted titanium dioxide photocatalysis on *E. coli* O157:H7, *L. monocytogenes*, and *Salmonella* Typhimurium, and observed severe damage on cell surface structures from the SEM images. Similar damage was also found on fungi and protozoa using titanium dioxide photocatalysis treatment (Pokhum, Viboonratanasri, & Chawengkijwanich, 2017). Furthermore, electrolyzed water containing highly oxidative chlorine compound was also demonstrated to be effective in destroying the protective barriers of *Bacillus subtilis* var. (Zeng et al., 2011) and *E. coli* O157:H7 (Nan et al., 2010). However, not all disinfection technologies inactivate pathogens through a similar mechanism. For example, Nicorescu et al. (2013) indicated pulsed UV light treatment can lead to 8 log reductions of *Bacillus subtilis* without affecting cell morphologies. They indicated the main mechanism was through direct DNA destruction.

5.4 Conclusions

Our study demonstrated ferrous and alkaline activated persulfate can cause cell injuries on *E. coli* O157:H7 and *L. monocytogenes.* Leakages of intracellular protein and DNA were found after activated persulfate treatment. The TTC-dehydrogenase relative activity of the treated pathogen cells was also significantly inhibited. SEM images show both activation treatments can result in cell surface structure damage, cell shrinkage, and hole formation. TEM images indicate cytoplasm started to agglutinate after 1 min alkaline activated persulfate treatment and the cell wall and cell membrane were severely damaged after 5 min treatment. Therefore, we propose the primary mechanism of ferrous and alkaline activated persulfate in inactivating *E. coli* O157:H7 and *L. monocytogenes* is through direct damage on cell envelope structures, leading to leakage of intracellular material and causing cytoplasm agglutination, and inhibition of key enzymes involved in metabolism, which finally result in cell death.

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Table 5.1. Surviving populations of *E. coli* O157:H7 and *L. monocytogenes* on non-selective and selective agar plates after activated persulfate treatment.

Treatment	Time (min)	Surviving population (log CFU/mL)			
		E. coli O157:H7		*L. monocytogenes*	
		TSASP	SMAC	BHIASP	LSA
Ferrous activation	0	9.28 ± 0.11^{Aa}	9.31 ± 0.10^{a}	9.59 ± 0.09^{Aa}	9.51 ± 0.05^{Aa}
	1	6.44 ± 0.27^{B}	ND	8.09 ± 0.24^{Ba}	6.70 ± 0.22^{Bb}
	2	4.96 ± 0.14^{C}	ND	6.78 ± 0.45^{Ca}	3.74 ± 0.27^{Cb}
	3	3.71 ± 0.49^{D}	ND	4.83 ± 0.55^{Da}	1.30 ± 0.30^{Db}
	4	2.61 ± 0.15^{E}	ND	2.83 ± 0.80^{Ea}	1.15 ± 0.21^{Db}
	5	ND	ND	1.67 ± 0.35^{F}	ND
Alkaline activation	0	9.35 ± 0.03^{Aa}	9.26 ± 0.06^{a}	9.48 ± 0.08^{Aa}	9.47 ± 0.09^{Aa}
	1	5.10 ± 0.17^{B}	ND	8.40 ± 0.10^{Ba}	8.20 ± 0.03^{Bb}
	2	3.57 ± 0.32^{C}	ND	5.95 ± 0.11^{Ca}	5.77 ± 0.15^{Ca}
	3	2.31 ± 0.92^{D}	ND	3.90 ± 0.18^{Da}	3.54 ± 0.13^{Db}
	4	1.63 ± 0.58^{DE}	ND	2.69 ± 0.43^{Ea}	2.47 ± 0.07^{Ea}
	5	1.45 ± 0.64^{E}	ND	1.75 ± 0.18^{Fa}	1.30 ± 0.30^{Fa}

Means (n = 3) in the same column under the same treatment group followed by the same uppercase letters are not significantly different ($P \geq 0.05$). Means in the same row under the same pathogen group followed by the same lowercase letters are not significantly different ($P \geq 0.05$). TSASP = tryptic soy agar supplemented with 0.1 % sodium pyruvate. SMAC = sorbitol MacConkey agar. BHIASP = brain heart infusion agar supplemented with 0.1 % sodium pyruvate. LSA = listeria selective agar supplemented with listeria selective supplement. ND =

not detected by direct plating. For ferrous activation, 40 mmol/L sodium persulfate and 13.3 mmol/L ferrous sulfate were used. For alkaline activation, 40 mmol/L sodium persulfate and 20 mmol/L sodium hydroxide was used to treat *E. coli* O157:H7 while 100 mmol/L sodium persulfate and 200 mmol/L sodium hydroxide was used to treat *L. monocytogenes*.

Table 5.2. DNA leakage of *E. coli* O157:H7 and *L. monocytogenes* after activated persulfate treatment.

Time (min)	DNA leakage (µg/mL)			
	E. coli O157:H7		*L. monocytogenes*	
	Ferrous activation	Alkaline activation	Ferrous activation	Alkaline activation
0	2.00 ± 0.33^{B}	2.08 ± 0.10^{B}	1.90 ± 0.49^{B}	1.93 ± 0.61^{B}
1	4.37 ± 1.50^{A}	29.00 ± 2.47^{A}	2.58 ± 0.25^{AB}	3.92 ± 0.83^{A}
2	5.03 ± 0.85^{A}	29.02 ± 2.63^{A}	3.08 ± 0.79^{AB}	4.58 ± 1.58^{A}
3	4.62 ± 0.13^{A}	29.25 ± 2.86^{A}	2.85 ± 0.25^{AB}	4.07 ± 0.34^{A}
4	4.60 ± 0.26^{A}	28.72 ± 2.92^{A}	3.13 ± 0.80^{A}	4.40 ± 0.61^{A}
5	5.42 ± 0.54^{A}	28.40 ± 2.86^{A}	2.87 ± 0.58^{AB}	5.02 ± 0.81^{A}

Means (n = 3) in the same column followed by the same uppercase letters are not significantly different ($P \geq 0.05$). For ferrous activation, 40 mmol/L sodium persulfate and 13.3 mmol/L ferrous sulfate were used. For alkaline activation, 40 mmol/L sodium persulfate and 20 mmol/L sodium hydroxide was used to treat *E. coli* O157:H7 while 100 mmol/L sodium persulfate and 200 mmol/L sodium hydroxide was used to treat *L. monocytogenes*.

Table 5.3. Protein leakage of *E. coli* O157:H7 and *L. monocytogenes* after alkaline activated persulfate treatment.

Time (min)	Protein leakage (µg/mL)	
	E. coli O157:H7	*L. monocytogenes*
0	0.63 ± 0.30^{D}	0.53 ± 0.18^{B}
1	7.33 ± 0.89^{C}	2.65 ± 1.00^{A}
2	8.85 ± 0.95^{BC}	2.78 ± 1.07^{A}
3	10.32 ± 1.42^{AB}	2.67 ± 0.97^{A}
4	11.26 ± 1.81^{A}	2.94 ± 1.00^{A}
5	12.07 ± 1.67^{A}	2.82 ± 1.02^{A}

Means (n = 3) in the same column followed by the same uppercase letters are not significantly different ($P \geq 0.05$). For alkaline activation, 40 mmol/L sodium persulfate and 20 mmol/L sodium hydroxide was used to treat *E. coli* O157:H7 while 100 mmol/L sodium persulfate and 200 mmol/L sodium hydroxide was used to treat *L. monocytogenes*.

Table 5.4. Degradation of protein standard under different activated persulfate treatment.

	Time (min)	Protein (ug/mL)
Control	0	15.73 ± 0.91^{A}
40 mmol/L PS + 13.3 mmol/L Fe	1	10.74 ± 0.44^{B}
	3	9.71 ± 0.18^{B}
	5	8.35 ± 0.24^{C}
40 mmol/L PS + 20 mmol/L NaOH	1	15.52 ± 0.66^{A}
	3	15.49 ± 0.49^{A}
	5	15.45 ± 0.67^{A}
100 mmol/L PS + 200 mmol/L NaOH	1	15.44 ± 0.90^{A}
	3	15.16 ± 0.56^{A}
	5	15.45 ± 0.85^{A}

Means (n = 3) followed by the same uppercase letters are not significantly different ($P \geq 0.05$).

PS = persulfate.

Table 5.5. TTC-dehydrogenase relative activity of *E. coli* O157:H7 and *L. monocytogenes* after activated persulfate treatment.

Time (min)	TTC-dehydrogenase relative activity (%)			
	E. coli O157:H7		*L. monocytogenes*	
	Ferrous activation	Alkaline activation	Ferrous activation	Alkaline activation
0	100.00 ± 0.00^{A}	100.00 ± 0.00^{A}	100.00 ± 0.00^{A}	100.00 ± 0.00^{A}
1	71.59 ± 15.48^{B}	2.36 ± 1.97^{B}	2.77 ± 0.37^{B}	23.14 ± 15.93^{B}
2	39.79 ± 3.45^{C}	2.19 ± 1.71^{B}	3.08 ±2.80 ^{B}	2.75 ± 2.29^{C}
3	19.02 ± 7.68^{D}	1.44 ± 1.70^{B}	1.30 ± 0.87^{B}	1.56 ± 1.50^{C}
4	3.53 ± 5.13^{E}	0.52 ± 0.34^{B}	3.03 ± 3.54^{B}	2.96 ± 2.65^{C}
5	0.90 ± 0.47^{E}	0.29 ± 0.37^{B}	2.36 ± 2.71^{B}	2.92 ± 2.34^{C}

Means (n = 3) in the same column followed by the same uppercase letters are not significantly different ($P \geq 0.05$). For ferrous activation, 40 mmol/L sodium persulfate and 13.3 mmol/L ferrous sulfate were used. For alkaline activation, 40 mmol/L sodium persulfate and 20 mmol/L sodium hydroxide was used to treat *E. coli* O157:H7 while 100 mmol/L sodium persulfate and 200 mmol/L sodium hydroxide was used to treat *L. monocytogenes*.

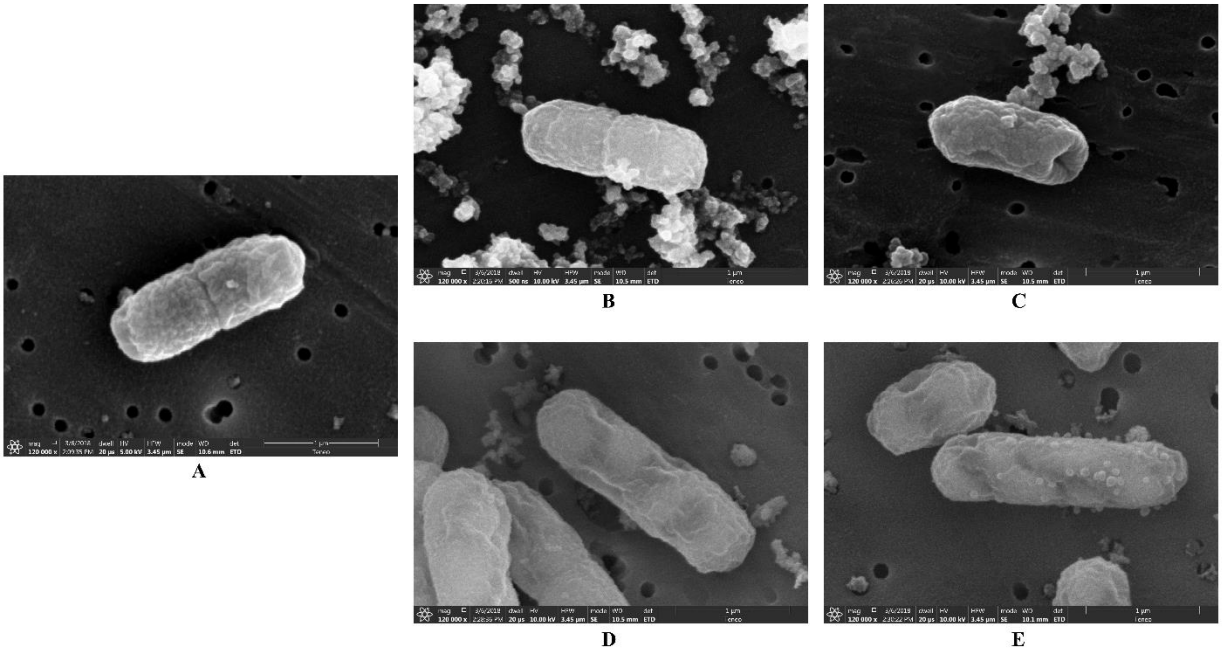

Fig. 5.1. SEM images of *E. coli* O157:H7: (A) untreated, (B) treated by ferrous activated persulfate for 1 min, (C) treated by ferrous activated persulfate for 5 min, (D) treated by alkaline activated persulfate for 1 min, (E) treated by alkaline activated persulfate for 5 min. For ferrous activation, 40 mmol/L sodium persulfate and 13.3 mmol/L ferrous sulfate were used. For alkaline activation, 40 mmol/L sodium persulfate and 20 mmol/L sodium hydroxide were used.

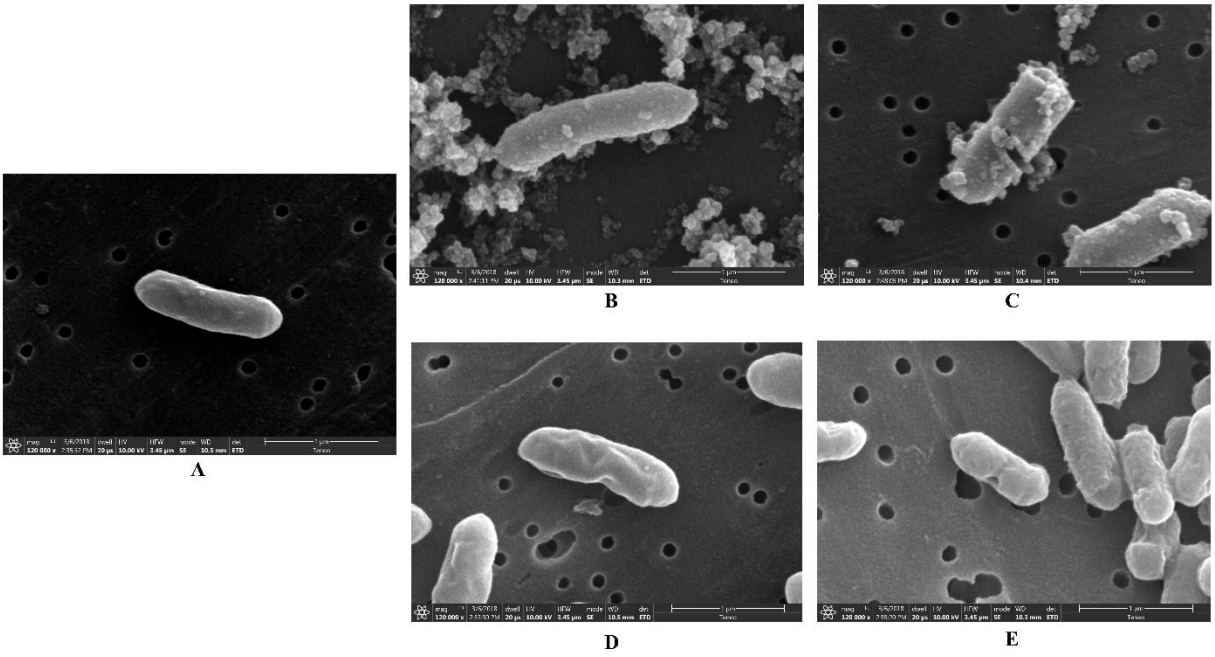

Fig. 5.2. SEM images of *L. monocytogenes*: (A) untreated, (B) treated by ferrous activated persulfate for 1 min, (C) treated by ferrous activated persulfate for 5 min, (D) treated by alkaline activated persulfate for 1 min, (E) treated by alkaline activated persulfate for 5 min. For ferrous activation, 40 mmol/L sodium persulfate and 13.3 mmol/L ferrous sulfate were used. For alkaline activation, 100 mmol/L sodium persulfate and 200 mmol/L sodium hydroxide were used.

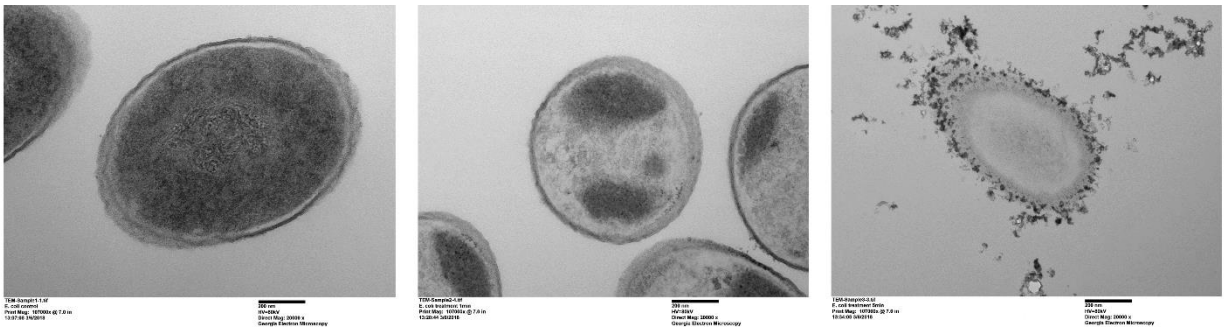

Fig. 5.3. TEM images of *E. coli* O157:H7: (A) untreated, (B) treated by alkaline activated persulfate for 1 min, (C) treated by alkaline activated persulfate for 5 min. The initial concentrations were 40 mmol/L sodium persulfate and 20 mmol/L sodium hydroxide.

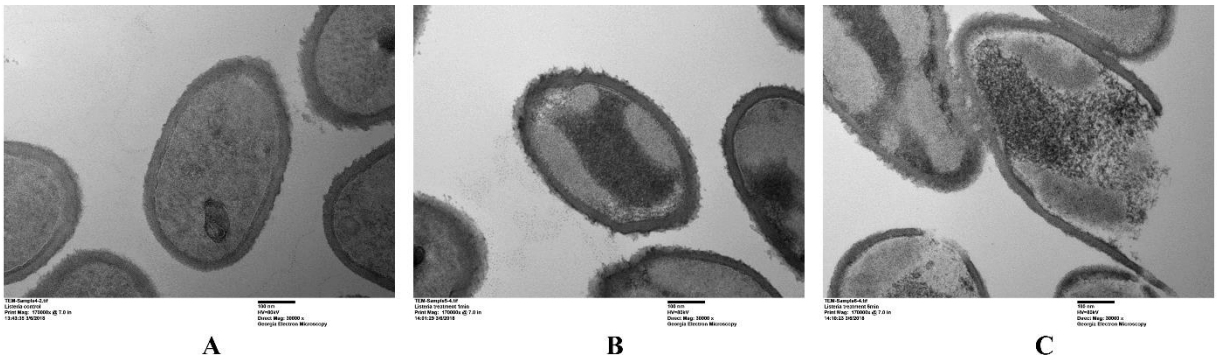

Fig. 5.4. TEM images of *L. monocytogenes*: (A) untreated, (B) treated by alkaline activated persulfate for 1 min, (C) treated by alkaline activated persulfate for 5 min. The initial concentrations were 100 mmol/L sodium persulfate and 200 mmol/L sodium hydroxide.

CHAPTER 6

REMOVAL OF FOODBORNE PATHOGENS FROM ROMAINE LETTUCE BY ACTIVATED PERSULFATE

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Abstract

Activated persulfate could be an alternative sanitizer for fresh produce decontamination. This study is the first in reporting the potential of activated persulfate in reducing *Escherichia coli* O157:H7 and *Listeria monocytogenes* from the surface of romaine. Ferrous sulfate and sodium hydroxide were used as the activators. Both activation methods achieved up to 3.5 log CFU/g reductions of *E. coli* O157:H7 and *L. monocytogenes* on romaine lettuce in 5min. Higher persulfate level and longer treatment time led to higher pathogen reductions. The wash water after each treatment was also collected and analyzed. *E. coli* O157:H7 survived in the wash water for both activation methods except when persulfate concentration reached 70 mmol/L. *L. monocytogenes* survived under all treatment conditions except for alkaline activation with persulfate concentration at 700 mmol/L. This might be due to the higher resistance of *L. monocytogenes* against activated persulfate treatment. Furthermore, both activation methods showed decreased efficacy in pathogen removal at refrigerated temperatures. However, they achieved equal or higher reduction efficacies than electrolyzed oxidizing water (100 mg/L), NaOCl (100 mg/L), and peroxyacetic acid (78 mg/L) at both 20 and 4 ^{o}C. In addition, under current test conditions, no significant color deterioration was found on activated persulfate treated lettuce.

Keywords

Activated persulfate; *E. coli* O157:H7; *L. monocytogenes*; Produce safety; Free radicals

6.1 Introduction

The nutritional value of fresh produce has been widely recognized by modern consumers and fresh produce has become a major component of healthy diet (Feng, et al., 2018; Meireles, Giaouris, & Simoes, 2016). As a result, the fresh produce market and industry have been increasing rapidly since the 1990s (Olaimat & Holley, 2012). However, foodborne outbreaks associated with consumption of fresh produce also increased from 0.7 % in the 1970s to 14.8% in 2007 (Sivapalasingam, Friedman, Cohen, & Tauxe, 2004). The U.S. CDC has reported 27% of outbreaks and 40% of foodborne illnesses were caused by the consumption of fresh produce in 2014 (CDC, 2014). Fresh produce-related outbreaks were estimated to cause over $1.4 billion annual economic losses in the U.S. (Batz, Hoffmann, & Morris, 2011). Foodborne pathogens, such as *Escherichia coli* O157:H7 and *Listeria monocytogenes*, are often associated with fresh produce-related outbreaks and illnesses (Yeni, Yavas, Alpas, & Soyer, 2016). Since fresh produce are often minimally processed and consumed raw, they can pose great health risks to consumers once contaminated (Olaimat & Holley, 2012). Therefore, it is of paramount importance for the fresh produce industry to effectively clean and sanitize harvested produce to eliminate potential pathogens (Gil, Gomez-Lopez, Hung, & Allende, 2015).

In the fresh produce industry, submerging harvested produce into a water-containing washing tank is the most commonly used processing treatment to remove contaminants, such as dirt and debris (Artes, Gomez, Aguayo, Escalona, & Artes-Hernandez, 2009). In order to further clean and sanitize fresh produce, a second washing tank containing sanitizing chemicals is often applied. In the U.S., chlorine-based sanitizers are most commonly used (Feliziani, Lichter, Smilanick, & Ippolito, 2016). Although chlorine-based sanitizers are highly effective in pathogen inactivation, there have been concerns regarding the potential to produce toxic disinfection by-

products (DBPs) when reacting with organic matter from produce (Joshi, Mahendran, Alagusundaram, Norton, & Tiwari, 2013). Because of the potential health risks of DBPs, chlorine-based sanitizers have been banned by many European countries for fresh produce washing (Van Haute, Sampers, Holvoet, & Uyttendaele, 2013). To minimize and avoid toxic DBPs formations, several alternative sanitizers have been studied, including peroxyacetic acid (PAA) (Singh, Hung, & Qi, 2018), electrolyzed water (Afari, Hung, King, & Hu, 2016), ozonated water (Selma, Allende, López-Gálvez, Conesa, & Gil, 2008), and hydrogen peroxide (Huang, Ye, & Chen, 2012). On the other hand, free radical-based advanced oxidation process (AOP), such as activated sodium persulfate, has been demonstrated as an effective antimicrobial treatment.

Sodium persulfate ($Na_2S_2O_8$) is a white power, stable at room temperature, and readily soluble in water (Matzek & Carter, 2016). Sodium persulfate is not a reactive chemical but can be activated by certain activators in water solution to produce reactive free radicals (Tsitonaki, et al., 2010). Ferrous sulfate and sodium hydroxide are the two common activators for persulfate activation (Matzek & Carter, 2016). Hydroxyl and sulfate radicals are the primary free radicals in ferrous activated persulfate (Liang, Bruell, Marley, & Sperry, 2004) while sulfate, hydroxyl, and superoxide radicals are the primary free radicals in sodium hydroxide activated persulfate (Furman, Teel, & Watts, 2010). Due to the highly oxidative and reactive power of these free radicals, activated persulfate has been used to degrade many recalcitrant environmental pollutants, such as atrazine (Hussain, Zhang, & Huang, 2014) and dinitrotoluenes (Chen, Jhou, & Huang, 2014). Recent studies have also found activated persulfate can be used for microbial inactivation, including *E. coli* O157:H7 (Qi, Huang, & Hung, 2018; Wordofa, Walker, & Liu, 2017), *L. monocytogenes* (Qi, et al., 2018), and *Bacillus* spores (Sun, Corey, & Huang, 2016).

However, no studies have validated the effect of activated persulfate in removing foodborne pathogens from fresh produce.

The current study aimed to study the effect of ferrous and alkaline activated persulfate in removing foodborne pathogens from the surface of romaine lettuce. The effects of initial persulfate concentration and treatment time were evaluated. The pathogen removal efficacy was also compared with other common sanitizing chemicals. In addition, the effect of activated persulfate on lettuce color quality was evaluated.

6.2 Materials and methods

6.2.1 Preparation of inoculum

Nalidixic acid adapted *E. coli* O157:H7 strains 1 (beef isolate), 4 (human isolate), 5 (human isolate), E009 (beef isolate), and 932 (human isolate), and *L. monocytogenes* strains Scott A (human isolate), LCDC 81-861 (raw cabbage isolate), F8027 (celery isolate), H77-50 (hot dog isolate), and F8369 (corn isolate) stored at -70 ^{o}C were used. *E. coli* O157:H7 strains were recovered by sub-culturing in tryptic soy broth (TSB, Difco, Sparks, MD, USA) and *L. monocytogenes* strains were sub-cultured in brain heart infusion broth (BHIB, Difco, Sparks, MD, USA), both at 37 ^{o}C for 24 h. Then, the cultured broths were sub-cultured in nalidixic acid (50 mg/L) supplemented TSB (TSBNA) for *E. coli* O157:H7 or BHIB (BHIBNA) for *L. monocytogenes* at 37 ^{o}C for 24 h. The final cultures were confirmed by streaking on MacConkey agar (Criterion, Santa Maria, CA, USA) (for *E. coli* O157:H7) or Palcam Listeria selective agar (Bioworld, Dublin, OH, USA) supplemented with Palcam Listeria selective supplement (EMD Millipore Corporation Billerica, MA, USA) (for *L. monocytogenes*) followed by incubation at 37 ^{o}C for 24 h and 48 h, respectively. Then, the final cultures were used as stock cultures. To

prepare the inoculum, the stock cultures were sub-cultured in TSBNA or BHIBNA for 24 h at 37 ^{o}C followed by centrifugation at 3000×g for 12 min. The cell pellets were washed with 20 mmol/L phosphate buffer saline (PBS, pH 7) and re-suspended in PBS. Each pathogen inoculum was made by combining equal volumes of the five strains and the final population of each pathogen inoculum was around 9.5 log CFU/mL.

6.2.2 Romaine lettuce inoculation

Fresh romaine lettuce was purchased from a local grocery store and kept at 4 ^{o}C until use (within 48 h). The outer two or three layers of leaves were discarded. The next layers of leaves without any obvious physical damage were selected. The selected leaves were trimmed to 20 ± 2 g and placed on sanitized trays with the abaxial side facing upwards. Two hundred microliters of the pathogen inoculum were inoculated on the top 2/3 area of each individual leaf by depositing tiny drops with a 200 µL-micropipette. All inoculated leaves were kept in a bio-safety hood for 1 h to allow pathogen attachment. Then all leaves were collected in a plastic sampling bag and stored overnight at 4 ^{o}C before washing treatment.

6.2.3 Washing treatment

A 2-L glass beaker was used as the washing container. Ferrous sulfate working solution (200 mmol/L) was freshly prepared each day. The beaker was first filled with pre-calculated amount of deionized (DI) water and sodium persulfate (Sigma-Aldrich, St. Louis, MO, USA). A spinning stir bar was placed inside the beaker to keep the solution homogenized. Two inoculated lettuce leaves were placed into the solution. Then, pre-calculated amount of the ferrous sulfate working solution or 10 mol/L sodium hydroxide solution (Fisher Scientific, Fair Lawn, NJ, USA) was added to the beaker to start the treatment. The total volume of the final solution was 1.5 L. After the designated treatment time, the leaves were immediately transferred to another

clean beaker with 1.5 L DI water and soaked for 30 s to remove residual chemicals from the surface. Each treated lettuce leaf was then transferred to a stomacher bag containing 180 mL neutralizing buffer (containing 1×strength Dey-Engley neutralizing broth, 20 mmol/L PBS, and 5 g/L sodium thiosulfate) and pummeled at 230 rpm for 2 min in a stomacher (Stomacher 80, Seward USA, Davie, FL, USA). Three initial persulfate levels (10, 40, and 70 mmol/L for *E. coli* O157:H7 and *L. monocytogenes* with ferrous activation or 100, 400, and 700 mmol/L for *L. monocytogenes* with alkaline activation) and three treatment times (1, 3, and 5 min) were tested. Immediately after each washing treatment, 20 mL of the remaining wash solution was collected and transferred to a 50-mL centrifuge tube containing 20 mL double-strength TSB (for *E. coli* O157:H7) or BHIB (for *L. monocytogenes*) supplemented with 0.2% sodium pyruvate, 200 mmol/L PBS and 20 g/L sodium thiosulfate. All washing treatments were conducted at 20 ^{o}C.

Efficacy of activated persulfate was also compared with other common sanitizers, including electrolyzed oxidizing (EO) water (100 mg/L free chlorine and pH 6.5), PAA (78 mg/L), NaOCl (100 mg/L free chlorine and pH 6.5), sodium hydroxide solution (200 mmol/L), ferric sulfate solution (23.3 mmol/L), and DI water. The condition selected for activated persulfate treatment were 70 mmol/L persulfate and 23.3 mmol/L ferrous sulfate, and 70 mmol/L persulfate and 20 mmol/L sodium hydroxide (*E. coli* O157:H7) or 700 mmol/L persulfate and 200 mmol/L sodium hydroxide (*L. monocytogenes*). All treatments were conducted for 5 min at 20 ^{o}C and 4 ^{o}C. EO water, PAA, and NaOCl were prepared using our previous method (Singh, et al., 2018). For 4 ^{o}C treatment, EO water was made a day before the experiment and stored in a capped glass container at 4 ^{o}C overnight. The rest of the solutions were freshly made on each experiment day using 4 ^{o}C DI water.

6.2.4 Microbiology analysis

The surviving pathogen population on lettuce was determined by plating 0.1 mL of 10-fold serial diluted pummeled solution onto respective agar plates. *E. coli* O157:H7 was plated on both tryptic soy agar supplemented with 50 mg/L nalidixic acid and 0.1 % sodium pyruvate (TSANASP) and sorbitol MacConkey agar supplemented with 50 mg/L nalidixic acid and 0.1 % sodium pyruvate (SMACNASP). *L. monocytogenes* was plated on both brain heart infusion agar supplemented with 50 mg/L nalidixic acid and 0.1 % sodium pyruvate (BHIANASP) and Palcam *Listeria* selective agar supplemented with 50 mg/L nalidixic acid and 0.1 % sodium pyruvate (LSANASP). Plates for *E. coli* O157:H7 were incubated at 37 ^{o}C for 24 h while plates for *L. monocytogenes* were incubated at 37 ^{o}C for 48 h. Two different types of agar plates were used for each pathogen because some background bacteria on lettuce are able to form colonies on TSANASP or BHIANASP while injured pathogen cells may not be able to form colonies on SMACNASP or LSANASP. In addition, the collected washing solutions were incubated at 37 ^{o}C for 24 h and streaked on the respective selective agars.

6.2.5 Color measurement of lettuce leaves

Color measurement was only conducted on activated persulfate treatment conditions that were used to compare with NaOCl, and DI water treatment at 20 ^{o}C as described in section 6.2.3. Lettuce leaves were selected with the same standard as described in section 6.2.2 without pathogen inoculation. The color of each leaf was first measured with a colorimeter (Model # CR-20, Konica Minolta, Inc., Japan) using the CIE color coordinates (L^*, a^*, b^*). The color measurement was conducted on three different spots on the abaxial side of lettuce leaves. All leaves were then treated following the treatment procedure as in described section 6.2.3. After

the treatment, each treated leaf was measured with the colorimeter again. Then, the total color difference (ΔE) for each leaf was calculated as $(\Delta a^{*2} + \Delta b^{*2} + \Delta L^{*2})^{1/2}$.

6.2.6 Statistical analysis

The entire experiment was repeated three times. Means and standard deviations were calculated in Excel (Micro software, USA). SAS software 9.4 (SAS Institute Inc., Cary, NC, USA) was used to conduct one-way analysis of variance and Duncan's multiple range test using proc glm procedure. Significant difference was considered at $P < 0.05$.

6.3 Results and discussion

6.3.1 Effectiveness of ferrous activated persulfate

The treatment conditions were based on our previous study (Qi, et al., 2018). The treatment time was controlled within 5 min because produce are usually washed for a few minutes in industrial practice (Martin-Belloso & Soliva-Fortuny, 2011). Two types of agar plates were used for *E. coli* O157:H7 (TSANASP and SMACNASP) and *L. monocytogenes* (BHIANASP and LSANASP). TSANASP and BHIANASP are relatively non-selective that some lettuce background bacteria are able to form colonies on while SMACNASP and LSANASP are selective such that background bacterial and inoculated pathogen injured cells may not be able to form colonies (Back, Kim, Park, Chung, & Kang, 2012). Also, the wash water after each treatment was collected and enriched to indicate cross-contamination potentials.

E. coli O157:H7 reductions after ferrous activated persulfate treatment are shown in Table 6.1. The overall reductions ranged from 1.63 to 3.34 log CFU/g and 2.42 to 3.65 log CFU/g for TSANASP and SMACNASP, respectively. The reduction trends as affected by the initial persulfate concentration and treatment time for both plates followed the same pattern. In

general, log reductions achieved using selective plates (SMACNASP) were about 0.5 log CFU/g higher than that of non-selective plates (TSANASP). Similar results were also found in Tables 6.2-6. Therefore, average reductions of these two types of plates were used for the following discussions.

From Table 6.1, both persulfate concentration and treatment time were important factors in *E. coli* O157:H7 reduction. Higher persulfate concentration and treatment time led to higher reductions. At persulfate concentration of 10 mmol/L, no significant ($P \geq 0.05$) increase of reduction was observed (2.02 to 2.35 log CFU/g reductions) when the treatment time was increased from 1 to 5 min. Significantly ($P < 0.05$) higher *E. coli* O157:H7 reductions were observed at 40 and 70 mmol/L persulfate when the treatment time was increased from 1 to 5 min (2.30 to 3.11 log CFU/g and 2.61 to 3.50 log CFU/g, respectively). On the other hand, no significantly ($P \geq 0.05$) higher reduction was observed when the persulfate concentration was increased from 10 to 40 mmol/L and from 40 to 70 mmol/L for each respective treatment time level, except for persulfate from 10 to 40 mmol/L with 5 min treatment (2.35 to 3.11 log CFU/g reductions). However, significantly ($P < 0.05$) higher reductions were always achieved when persulfate was increased from 10 to 70 mmol/L at each comparable treatment time level (Table 6.1). In addition, the wash water enrichment were all positive for the 10 mmol/L persulfate treatment. After persulfate was increased to 40 mmol/L, the wash water enrichment were partially positive for 1 and 3 min treatment times and completely negative after 5 min treatment. This indicates enough treatment time is required for 40 mmol/L persulfate to completely inactivate the pathogen cells migrated into the wash water. Persulfate at 70 mmol/L, on the other hand, showed negative enrichment in wash water at all treatment time levels, indicating its high potential in preventing cross-contamination.

The reductions of *L. monocytogenes* after ferrous activated persulfate treatment are shown in Table 6.2. The reductions ranged from 1.20 to 3.50 log CFU/g with the maximum reduction achieved at 70 mmol/L persulfate after 5 min. Similar to *E. coli* O157:H7, both persulfate concentration and treatment time had significant impacts on the reductions. The extension of treatment time from 1 to 5 min significantly ($P < 0.05$) increased the *L. monocytogenes* reductions from 1.20 to 1.94 log CFU/g, 1.75 to 2.95 log CFU/g, and 2.34 to 3.50 log CUF/g at 10, 40, and 70 mmol/L persulfate, respectively. Also, significant ($P < 0.05$) improvement of reductions were observed by increasing the persulfate concentration from 10 to 40 mmol/L and 40 to 70 mmol/L at each comparable treatment time level (Table 6.2). However, the enrichment results were all positive at all treatment combinations, except for the treatment with 70 mmol/L persulfate and 5 min treatment time where one out of three replications showed negative enrichment. This indicates the *L. monocytogenes* cells washed from lettuce surface were not completely inactivated and could resuscitate at desired conditions. This result is in accordance with our previous study (Qi, et al., 2018) that *L. monocytogenes* is more resistant against ferrous activated persulfate treatment than *E. coli* O157:H7.

No report in the literature has evaluated the potential of ferrous activated persulfate for fresh produce sanitation. Most microbial studies using activated persulfate focused on alleviating environmental microbial contaminations. For example, Samyoung, Peterson, Righter, Miles, and Tratnyek (2013) demonstrated iron activated persulfate were able to effectively reduce the viability and photosynthetic performance of *Pseudonitzshia delicatissima* and green alga *Dunaliella tertiolecta* in ballast water. Wang, Chen, Xie, Shang, and Ma (2016) also indicated that *Microcystis aeruginosa* related cyanobacteria bloom in source water can be dramatically improved with UV light-activated persulfate. However, these environmental applications were

conducted for hours and days at low persulfate levels (around 5 mmol/L), which are not applicable for fresh produce sanitation. The current study demonstrated efficient pathogen reduction on romaine lettuce can be achieved in only a few minutes by using high persulfate concentrations (> 40 mmol/L).

6.3.2 Effectiveness of alkaline activated persulfate

The treatment conditions were selected based on our previous study (Qi, et al., 2018). The results of *E. coli* O157:H7 reductions are shown in Table 6.3, ranging from 2.33 to 3.41 log CFU/g. The maximum reduction was achieved at the highest persulfate level and longest treatment time (70 mmol/L for 5 min). The increase of treatment time from 1 to 5 min resulted in significantly ($P < 0.05$) higher *E. coli* O157:H7 reductions at 10 and 70 mmmol/L persulfate (2.34 to 3.02 log CFU/g reductions and 2.80 to 3.41 log CFU/g reductions, respectively). However, no significantly ($P \geq 0.05$) higher reduction was observed at 40 mmol/L persulfate (2.60 to 2.98 log CFU/g reductions). On the other hand, the increase of persulfate from 10 to 40 mmol/L and 40 to 70 mmol/L achieved more reductions at each comparable treatment time level (Table 6.3). However, most of the increases were not significant ($P \geq 0.05$). Although the effect of persulfate level and treatment time on *E. coli* O157:H7 reductions was not very obvious, their influence on wash water is significant. As Table 6.3 shows, 10 mmol/L persulfate treatment showed all positive enrichment results, indicating strong potentials for cross-contamination. The increase of persulfate to 40 and 70 mmol/L dramatically decreased the enrichment positives that 40 mmol/L persulfate only showed partially positive enrichment and 70 mmol/L showed complete negative enrichment. This indicates the necessity to have enough persulfate level for alkaline activation treatment to prevent cross-contamination.

A different sodium hydroxide level (200 mmol/L) and persulfate concentration range (100 to 700 mmol/L) were used to treat lettuce inoculated with *L. monocytogenes*. The condition was also based on our previous study (Qi, et al., 2018). The reductions were ranged from 1.32 to 3.57 log CFU/g (Table 6.4). Significant effect of persulfate level and treatment time were observed. At each persulfate level, the increase of treatment time from 1 to 3 min always led to significantly ($P < 0.05$) higher reductions (on average about 0.8 log CFU/g reduction increase). However, further increase from 3 to 5 min achieved only about 0.5 log CFU/g more reduction at each persulfate level and the differences were not significant ($P \geq 0.05$). The persulfate concentration effect was not very obvious from 100 to 400 mmol/L and from 400 to 700 mmol/L. Significantly ($P < 0.05$) higher reductions were only observed between 100 and 700 mmol/L persulfate where at least 0.6 log CFU/g higher reductions was obtained. For the wash water enrichment test, complete positive results were found at the low persulfate level (100 mmol/L), partially positive results were found at the medium persulfate level (400 mmol/L), and complete negative results were found at the high persulfate level (700 mmol/L). This indicates the necessity of enough persulfate or treatment time period for complete inactivation of *L. monocytogenes* in wash water.

6.3.3 Comparison of activated persulfate with other sanitizers in pathogen reduction

The efficacy of activated persulfate was compared with other common sanitizers, including electrolyzed water, NaOCl, and PAA. DI water was selected as a negative control treatment. Ferric sulfate and sodium hydroxide were also selected because they are either the final activation product (ferrous activation) or the activator (alkaline activation) and demonstrated some antimicrobial effect (Qi, et al., 2018). The comparison study was conducted at both 20 ^{o}C and 4 ^{o}C because using wash water at low temperature is a common practice in the

fresh produce industry. Table 6.5 shows the reduction comparisons on *E. coli* O157:H7. At 20 ^{o}C, the two activated persulfate treatments and EO water achieved significantly ($P < 0.05$) higher reductions of *E. coli* O157:H7 than the other treatments. Although PAA and NaOCl only obtained about 2.90 log CFU/g reduction, they were still more effective than sodium hydroxide (2.45 log CFU/g reductions) and ferric sulfate (2.31 log CFU/g reductions). Also, even though PAA and NaOCl treatment did not demonstrate strong pathogen reduction efficacy, they achieved complete negative enrichment results, indicating the ability to prevent cross-contamination. On the other hand, both sodium hydroxide and ferric sulfate showed all positive in wash water enrichment, indicating their limited antimicrobial effect. DI water was the least effective treatment in *E. coli* O157:H7 reduction (1.24 log CFU/g). On the other hand, when the treatment solution temperature was reduced to 4 ^{o}C, a lower reduction was observed in all treatments. The efficacy of the two activated persulfate treatments reduced to about 2.9 log CFU/g reductions but they were still more effective than the other six treatments. Although the reduction efficacy was decreased at lower temperature, the wash water still showed complete negative enrichment results for activated persulfate, EO water, and NaOCl, except PAA where partial positive enrichment was observed.

Similarly, both ferrous (3.50 log CFU/g reductions) and alkaline (3.57 log CFU/g reductions) activated persulfate treatment showed higher reductions than other treatments on *L. monocytogenes* at 20 ^{o}C (Table 6.6). About 3 log CFU/g reductions were observed in EO water, NaOCl, and PAA. The sodium hydroxide and ferric sulfate treatments only achieved about 2 log CFU/g *L. monocytogenes* reductions. Only alkaline activated persulfate showed all negative enrichment while ferrous activated persulfate, EO water, NaOCl, and PAA showed partial positive enrichment. This might be due to the much higher initial persulfate level of alkaline

activation (Table 6.4) used for *L. monocytogenes* that has more free radicals and higher antimicrobial power (Qi, et al., 2018). At 4 ^{o}C, lowered reductions were also observed. All sanitizers achieved reductions below 3 log CFU/g. The two activated persulfate treatments achieved about 2.8 log CFU/g reductions at 4 ^{o}C which are significantly ($P < 0.05$) lower than that at 20 ^{o}C. However, at 4 ^{o}C, the two activated persulfate treatments still showed higher reductions than the other treatments. In addition, only the wash water of alkaline activated persulfate treatment showed complete negative enrichment results while the other treatments showed partial or complete positive enrichment results.

The temperature effect from the comparison studies can be explained by two possible theories. Firstly, the wash water temperature is suggested to be at least 10 ^{o}C higher than that of fresh produce to prevent temperature pressure differential (Parish, et al., 2003). Otherwise, the pathogens on produce surface may be infiltrated to the interior parts. In our study, both the produce and wash solutions were kept at 4 ^{o}C which does not meet the temperature difference suggestion. Therefore, pathogen infiltration during the treatment was possible so that fewer reductions were observed at 4 ^{o}C. Secondly, the efficacy of sanitizing chemicals can be temperature dependent (i.e. lower temperature will lead to lower efficacies). For example, both chlorine dioxide and ozone were found to have lower removal efficacy at lower temperature (5 – 30 ^{o}C) against *E. coli* O157:H7, *Pseudomonas aeruginosa*, *Mycobacterium avium*, and *Bacillus subtilis* spores (Larson & Mariñas, 2003; Taylor, Rogers, & Holah, 1999; Vicuña-Reyes, Luh, & Mariñas, 2008). This temperature dependency might be different between different sanitizers since EO water showed great reduction decrease (0.5 – 1 log CFU/g) at lower temperature while PAA showed minor reduction decrease (0.2 – 0.3 log CFU/g). In addition, the reactivity of free radical are also temperature dependent. The reaction rate between hydroxyl radicals with

chloramine and organic matter were found to be reduced at lower temperature (Gleason, McKay, Ishida, & Mezyk, 2017; McKay, Dong, Kleinman, Mezyk, & Rosario-Ortiz, 2011). As free radicals, such as hydroxyl radicals, were demonstrated to be the primary compounds in pathogen inactivation (Qi, et al., 2018), the lower pathogen removal efficacy on romaine lettuce shown in the current study can be explained by the lower reactivity of free radicals.

Furthermore, the reduction results from the current study were also comparable with the reductions achieved by studies using other alternative sanitizers on leafy green products. Singh, Singh, Bhunia, and Stroshine (2002) studied the effect of chlorine dioxide (10 mg/L) in reducing *E. coli* O157:H7 from fresh lettuce and achieved 1.2 log CFU/g reductions in 5 min. Karaca and Velioglu (2014) achieved 2.0 log CFU/g and 2.2 log CFU/g reductions of *E. coli* Type 1 and *L. innocua* on fresh lettuce in 15 min, respectively, using 12 mg/L ozonated water. In addition, the combination of 3 mg/L ozonated water and 1% citric acid was demonstrated to achieve 2.31 log CFU/g and 1.84 log CFU/L reductions of *E. coli* O157:H7 and *L. monocytogenes* on iceberg lettuce, respectively (Yuk, et al., 2006). Our activated persulfate treatment achieved about 2.5 to 3.5 log CFU/g reductions of *E. coli* O157:H7 and *L. monocytogenes* from romaine lettuce in 5 min, which are comparable with the efficacy of the aforementioned alternative sanitizers for lettuce sanitation.

6.3.4 Effect of activated persulfate treatment on lettuce color quality

One potential concern of activated persulfate treatment is the effect on produce quality. For ferrous activation, the final activation product is ferric sulfate which is a red color in water solution. This might lead to significant color change on produce. For alkaline activation, the use of sodium hydroxide might also have strong influence on produce color quality due to high alkalinity. The effect of activated persulfate treatment on lettuce color quality was evaluated

using the maximum persulfate concentration (70 mmol/L for ferrous activation and 700 mmol/L for alkaline activation) and 5 min treatment time at 20 ^{o}C. It is generally agreed that wash water at lower temperature would better preserve the produce quality. Therefore, the color effect was not evaluated at 4 ^{o}C. In addition, only NaOCl and DI water were tested as comparisons because it is well documented that chlorine-based sanitizers and PAA would not cause significant quality deterioration on fresh produce (Bang, Park, Kim, Rahaman, & Ha, 2017; Gil, et al., 2015). The results of lettuce color before and after the treatment are shown in Table 6.7. Ferrous activated persulfate caused more changes on both *a** (0.5 difference) and *b** (0.6 difference), while the other three treatments only showed minor changes on *a** (less than 0.1 difference) and *b** (less than 0.3 difference). However, the total color difference of all four treatments are all below 1.5, which fall into the category of small difference (Pathare, Opara, & Al-Said, 2013). That indicates ferrous and alkaline activated persulfate treatment will not cause significant color change on romaine lettuce under our current test conditions.

6.4 Conclusions

The current study demonstrated that both ferrous and alkaline activated persulfate can effectively reduce *E. coli* O157:H7 and *L. monocytogenes* from romaine lettuce. Higher persulfate concentration and longer treatment time can lead to higher reductions. In addition, cross-contamination of pathogens through wash water can be minimized by using appropriate persulfate concentration and treatment time. The activated persulfate treatment was also shown to be equally or more effective than chlorine-based sanitizers and PAA in pathogen reduction. Within the current test conditions, activated persulfate treatment would not cause significant changes on romaine lettuce color quality. Based on the overall results, activated persulfate

treatment can be used as an alternative sanitizer to replace traditional chlorine-based sanitizers in ensuring fresh produce safety.

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Table 6.1. Reduction of *E. coli* O157:H7 populations on romaine lettuce after treatment with ferrous activated persulfate.

Initial persulfate concentration (mmol/L)	Time (min)	Reduction (log CFU/g)			Wash Water[b]
		TSANASP	SMACNASP	Average[a]	
10	1	1.63 ± 0.24	2.42 ± 0.23	2.02 ± 0.21^{E}	3/3
	3	2.24 ± 0.27	2.36 ± 0.26	2.30 ± 0.23^{DE}	3/3
	5	2.23 ± 0.20	2.48 ± 0.35	2.35 ± 0.27^{DE}	3/3
40	1	2.10 ± 0.36	2.50 ± 0.37	2.30 ± 0.36^{DE}	1/3
	3	2.42 ± 0.36	2.97 ± 0.58	2.70 ± 0.46^{BCD}	2/3
	5	2.98 ± 0.16	3.25 ± 0.06	3.11 ± 0.10^{AB}	0/3
70	1	2.52 ± 0.29	2.70 ± 0.14	2.61 ± 0.21^{CD}	0/3
	3	2.64 ± 0.25	3.14 ± 0.08	2.89 ± 0.13^{BC}	0/3
	5	3.34 ± 0.08	3.65 ± 0.30	3.50 ± 0.17^{A}	0/3

[a]Mean values in this column followed by the same capital letter are not significantly different ($P \geq 0.05$).

[b]n = 0/3 means out of 3 replications, none wash water enrichment showed positive recovery of *E. coli* O157:H7.

The molar ratio of persulfate to ferrous was controlled at 3:1. The initial *E. coli* O157:H7 population on lettuce was 7.19 ± 0.25 log CFU/mL (average).

Table 6.2. Reduction of *L. monocytogenes* populations on romaine lettuce after treatment with ferrous activated persulfate.

Initial persulfate concentration (mmol/L)	Time (min)	Reduction (log CFU/g)			Wash Water[b]
		BHIANASP	LSANASP	Average[a]	
10	1	1.08 ± 0.25	1.34 ± 0.34	1.20 ± 0.25^{F}	3/3
	3	1.44 ± 0.15	1.60 ± 0.29	1.51 ± 0.15^{EF}	3/3
	5	1.79 ± 0.30	2.11 ± 0.45	1.94 ± 0.35^{DE}	3/3
40	1	1.58 ± 0.12	1.95 ± 0.09	1.75 ± 0.05^{E}	3/3
	3	2.26 ± 0.39	2.68 ± 0.41	2.46 ± 0.36^{C}	3/3
	5	2.78 ± 0.23	3.16 ± 0.24	2.95 ± 0.14^{B}	3/3
70	1	2.15 ± 0.39	2.56 ± 0.57	2.34 ± 0.46^{CD}	3/3
	3	2.98 ± 0.13	3.25 ± 0.16	3.10 ± 0.13^{AB}	3/3
	5	3.32 ± 0.18	3.71 ± 0.53	3.50 ± 0.28^{A}	2/3

[a]Mean values in this column followed by the same capital letter are not significantly different ($P \geq 0.05$).

[b]n = 0/3 means out of 3 replications, none wash water enrichment showed positive recovery of *L. monocytogenes*.

The molar ratio of persulfate to ferrous was controlled at 3:1. The initial *L. monocytogenes* population on lettuce was 7.60 ± 0.18 log CFU/mL (average).

Table 6.3. Reduction of *E. coli* O157:H7 populations on romaine lettuce after treatment with alkaline activated persulfate.

Initial persulfate concentration (mmol/L)	Time (min)	Reduction (log CFU/g)			Wash Water[b]
		TSANASP	SMACNASP	Average[a]	
10	1	2.31 ± 0.18	2.37 ± 0.22	2.34 ± 0.19^{C}	3/3
	3	2.14 ± 0.56	2.53 ± 0.28	2.33 ± 0.38^{C}	3/3
	5	2.94 ± 0.19	3.10 ± 0.19	3.02 ± 0.19^{AB}	3/3
40	1	2.69 ± 0.48	2.52 ± 0.30	2.60 ± 0.39^{BC}	2/3
	3	3.04 ± 0.07	3.04 ± 0.16	3.04 ± 0.11^{AB}	0/3
	5	2.96 ± 0.09	3.00 ± 0.14	2.98 ± 0.11^{AB}	1/3
70	1	2.79 ± 0.26	2.82 ± 0.35	2.80 ± 0.30^{BC}	0/3
	3	2.84 ± 0.15	3.00 ± 0.15	2.92 ± 0.01^{B}	0/3
	5	3.31 ± 0.33	3.50 ± 0.38	3.41 ± 0.34^{A}	0/3

[a]Mean values in this column followed by the same capital letter are not significantly different ($P \geq 0.05$).

[b]n = 0/3 means out of 3 replications, none wash water enrichment showed positive recovery of *E. coli* O157:H7.

The concentration of NaOH was controlled at 20 mmol/L. The initial *E. coli* O157:H7 population on lettuce was 7.34 ± 0.12 log CFU/mL (average).

Table 6.4. Reduction of *L. monocytogenes* populations on romaine lettuce after treatment with alkaline activated persulfate.

Initial persulfate concentration (mmol/L)	Time (min)	Reduction (log CFU/g)			Wash Water[b]
		BHIANASP	LSANASP	Average[a]	
100	1	1.44 ± 0.19	1.37 ± 0.19	1.32 ± 0.03^{F}	3/3
	3	2.53 ± 0.33	2.58 ± 0.48	2.47 ± 0.34^{CDE}	3/3
	5	2.90 ± 0.64	3.07 ± 0.68	2.90 ± 0.54^{BCD}	3/3
400	1	2.23 ± 0.41	2.29 ± 0.45	2.17 ± 0.26^{E}	2/3
	3	3.04 ± 0.39	3.10 ± 0.23	2.98 ± 0.16^{BC}	1/3
	5	3.07 ± 0.56	3.09 ± 0.64	2.99 ± 0.47^{BC}	0/3
700	1	2.32 ± 0.19	2.60 ± 0.44	2.37 ± 0.13^{DE}	0/3
	3	3.12 ± 0.42	3.18 ± 0.47	3.06 ± 0.33^{AB}	0/3
	5	3.58 ± 0.32	3.74 ± 0.11	3.57 ± 0.11^{A}	0/3

[a]Mean values in this column followed by the same capital letter are not significantly different ($P \geq 0.05$).

[b]n = 0/3 means out of 3 replications, none wash water enrichment showed positive recovery of *L. monocytogenes*.

The concentration of NaOH was controlled at 200 mmol/L. The initial *L. monocytogenes* population on lettuce was 7.57 ± 0.15 log CFU/mL (average).

Table 6.5. Comparison of different sanitizers on reduction of *E. coli* O157:H7 populations on romaine lettuce surface at 4 and 20 ºC.

Treatment	Reduction (log CFU/mL)[a]							
	20 ºC				4 ºC			
	TSANASP	SMACNASP	Average[a]	Wash Water[b]	TSANASP	SMACNASP	Average[a]	Wash Water[b]
Ferrous activation	3.34 ± 0.08	3.65 ± 0.30	3.50 ± 0.17^{Aa}	0/3	2.73 ± 0.07	2.92 ± 0.26	2.83 ± 0.15^{ABb}	0/3
Alkaline activation	3.31 ± 0.33	3.50 ± 0.38	3.41 ± 0.34^{Aa}	0/3	2.85 ± 0.31	3.16 ± 0.38	3.01 ± 0.35^{Aa}	0/3
EO water	3.34 ± 0.11	3.52 ± 0.14	3.51 ± 0.15^{Aa}	0/3	2.28 ± 0.29	2.38 ± 0.25	2.33 ± 0.26^{Cb}	0/3
PAA	2.87 ± 0.27	2.99 ± 0.29	2.93 ± 0.28^{Ba}	0/3	2.73 ± 0.11	2.79 ± 0.11	2.76 ± 0.11^{ABa}	2/3
NaOCl	2.80 ± 0.12	2.81 ± 0.17	2.81 ± 0.14^{BCa}	0/3	2.38 ± 0.30	2.48 ± 0.40	2.43 ± 0.35^{BCa}	0/3
NaOH	2.35 ± 0.36	2.51 ± 0.50	2.45 ± 0.45^{CDa}	3/3	2.21 ± 0.11	2.22 ± 0.29	2.22 ± 0.15^{Ca}	3/3
Ferric sulfate	2.12 ± 0.10	2.49 ± 0.18	2.31 ± 0.14^{Da}	3/3	2.07 ± 0.11	2.31 ± 0.22	2.19 ± 0.14^{Ca}	3/3
DI water	1.16 ± 0.26	1.34 ± 0.32	1.24 ± 0.31^{Ea}	3/3	1.18 ± 0.19	1.32 ± 0.27	1.25 ± 0.22^{Da}	3/3

[a]Mean values in the same column followed by the same capital letter are not significantly different ($P \geq 0.05$). Mean values in the same row followed by the same lowercase letter are not significantly different ($P \geq 0.05$).

[b]n = 0/3 means out of 3 replications, none wash water enrichment showed positive recovery of *E. coli* O157:H7.

For ferrous activation, 70 mmol/L initial persulfate and 23.3 mmol/L ferrous sulfate was used. For Alkaline activation, 70 mmol/L initial persulfate and 20 mmol/L NaOH was used. EO water and NaOCl were at 100 mg/L free chlorine and pH 6.5. PAA was at 78 mg/L. NaOH was at 20 mmol/L. Ferric sulfate was at 23.3 mmol/L. All treatment were conducted for 5 min.

Table 6.6. Comparison of different sanitizers on reduction of *L. monocytogenes* populations on romaine lettuce surface 4 and 20 °C.

Treatment	Reduction (log CFU/mL)[a]							
	20 °C				4 °C			
	BHIANASP	LSANASP	Average[a]	Wash Water[b]	BHIANASP	LSANASP	Average[a]	Wash Water[b]
Ferrous activation	3.32 ± 0.18	3.71 ± 0.53	3.50 ± 0.28^{Aa}	2/3	2.75 ± 0.21	2.75 ± 0.18	2.75 ± 0.19^{Ab}	1/3
Alkaline activation	3.58 ± 0.32	3.74 ± 0.11	3.57 ± 0.11^{Aa}	0/3	2.81 ± 0.35	2.81 ± 0.21	2.81 ± 0.28^{Ab}	0/3
EO water	2.95 ± 0.17	2.95 ± 0.15	2.95 ± 0.16^{Ba}	2/3	2.55 ± 0.26	2.41 ± 0.34	2.48 ± 0.30^{Aa}	2/3
PAA	3.10 ± 0.44	3.09 ± 0.49	3.09 ± 0.46^{ABa}	2/3	2.65 ± 0.10	2.68 ± 0.03	2.67 ± 0.06^{Aa}	2/3
NaOCl	3.16 ± 0.38	3.27 ± 0.36	3.21 ± 0.37^{ABa}	1/3	2.67 ± 0.38	2.62 ± 0.34	2.65 ± 0.36^{Aa}	3/3
NaOH	1.68 ± 0.38	1.85 ± 0.38	1.82 ± 0.44^{Ca}	3/3	2.12 ± 0.01	2.09 ± 0.05	2.11 ± 0.02^{Ba}	2/3
Ferric sulfate	2.06 ± 0.19	2.08 ± 0.09	2.06 ± 0.14^{Ca}	3/3	1.66 ± 0.01	2.00 ± 0.04	1.83 ± 0.02^{Ba}	3/3
DI water	0.94 ± 0.06	1.11 ± 0.10	1.04 ± 0.08^{Da}	3/3	1.11 ± 0.12	1.05 ± 0.01	1.08 ± 0.06^{Ca}	3/3

[a]Mean values in the same column followed by the same capital letter are not significantly different ($P \geq 0.05$). Mean values in the same row followed by the same lowercase letter are not significantly different ($P \geq 0.05$).

[b]n = 0/3 means out of 3 replications, none wash water enrichment showed positive recovery of *L. monocytogenes*.

For ferrous activation, 70 mmol/L initial persulfate and 23.3 mmol/L ferrous sulfate was used. For Alkaline activation, 700 mmol/L initial persulfate and 200 mmol/L NaOH was used. EO water and NaOCl were at 100 mg/L free chlorine and pH 6.5. PAA was at 78 mg/L. NaOH was at 200 mmol/L. Ferric sulfate was at 23.3 mmol/L. All treatment were conducted for 5 min.

Table 6.7. Lettuce color difference before and after different sanitizing treatment.

Treatment	Before[a]			After[a]			(ΔE^*)
	L^*	a^*	b^*	L^*	a^*	b^*	
Ferrous activated persulfate[b]	43.0	7.9	17.2	43.6	7.4	16.6	1.07 ± 0.53
Alkaline activated persulfate[c]	47.5	8.0	17.8	46.9	8.1	17.8	0.89 ± 0.13
NaOCl at 100 mg/L and pH 6.5	46.4	7.4	15.1	46.7	7.4	15.4	0.54 ± 0.46
DI water	46.5	7.6	15.0	46.2	7.6	14.8	0.76 ± 0.50

[a]Numbers under these columns are means of three replicates.

[b]70 mmol/L initial persulfate and 23.3 mmol/L ferrous sulfate.

[c]700 mmol/L initial persulfate and 200 mmol/L NaOH.

CHAPTER 7

EFFECT OF ORGANIC LOAD ON THE PATHOGEN INACTIVATION EFFICACY OF ACTIVATED PERSULFATE

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Abstract

Persulfate activated by alkaline reagents or transition metals is highly effective in eliminating foodborne pathogens. There is great potential to apply this novel sanitizing treatment in fresh produce sanitation. The current study was undertaken to evaluate the potential effect of organic load (fresh produce) on the pathogen inactivation efficacy of activated persulfate. Romaine lettuce, iceberg lettuce, strawberries, and grapes were used to prepare the produce extract and diluted to different organic load levels (Chemical oxygen demand (COD) at 100 to 2000 mg/L) to simulate the wash water property in produce processing. Ferrous activated persulfate (PS/Fe^{2+}) and alkaline activated persulfate (PS/OH^{-}) were spiked with the organic matter to treat a five-strain cocktail of *Escherichia coli* O157:H7 in aqueous solution for 2 min. Water properties including COD, phenolics, protein content, UV254 absorbance, turbidity, pH, and ORP were measured and correlated with the microbial reductions. Romaine lettuce inoculated with *E. coli* O157:H7 was used to validate the organic load effect. The results showed that by spiking the produce extract into activated persulfate solution, the *E. coli* O157:H7 inactivation was decreased from over 8.0 log CFU/mL to near 2 log CFU/mL. The organic load has a significantly larger effect on PS/Fe^{2+} than PS/OH^{-}. Based on the correlation coefficients, COD, phenolics, and UV254 were found to be the major indicators to predict the organic load effect. However, since UV254 measurement takes much shorter time, UV254 was considered as the primary indicator for real application. From the inoculated romaine lettuce washing study, lower reductions and high cross-contamination potentials were observed for PS/Fe^{2+}, PS/OH^{-}, and NaOCl treatments spiked with organic load (UV254 at 0.750) compared with the respective treatments without organic load. Therefore, it was concluded that to maintain the high efficacy of

activated persulfate for fresh produce washing, the organic load of wash water must be carefully monitored.

Keywords

Activated persulfate, *Escherichia coli* O157:H7, Chemical oxygen demand, UV254, Fresh produce

7.1 Introduction

Consumption of sufficient fresh produce in daily diets has been considered as the key to maintain good health (DeFosset, Gase, Webber, & Kuo, 2017). However, fresh produce can also pose potential threats as they may be contaminated with many foodborne pathogens and have been associated with many foodborne outbreaks (Goodburn & Wallace, 2013; Lynch, Tauxe, & Hedberg, 2009). From 1998 to 2008, more than 9 million people suffered from foodborne illnesses in the U.S., in which 46% were attributed to the consumption of fresh produce (Painter, et al., 2013). Many risk factors can contribute to the produce contamination, such as fecal contaminated soil, fecal contaminated irrigation water, and poor handling hygiene during postharvest processing (Nguyen-the, et al., 2016). Since there is no kill step in fresh produce processing, pathogens attached on fresh produce can survive postharvest (Luo, et al., 2018). Currently, the most widely used practice in the fresh produce industry is to wash the harvested produce in a washing tank to remove physical contaminants, such as dirt and debris (Artes, Gomez, Aguayo, Escalona, & Artes-Hernandez, 2009). The washing process will not only remove those physical contaminants but also dislodge the pathogens attached on produce, hence can allow pathogens to transfer to other produce through the washing solution (Luo, et al., 2018). Therefore, the addition of sanitizing chemicals is critical to prevent the potential of cross-contamination as well as reduce the load of pathogen levels on produce.

In the U.S., chlorine (e.g. sodium hypochlorite) is the most commonly used sanitizing chemical in the fresh produce industry because of the low price and high efficacy (Chen & Hung, 2017; Gombas, et al., 2017). However, recent studies have indicated harmful chlorinated disinfection by-products (DBPs) can be generated when chlorine reacts with organic matter from produce (Fan & Sokorai, 2015). Trihalomethanes (THMs) and haloacetic acids (HAAs) are the

two common DBPs found in chlorinated waters and they are toxic and potentially carcinogenic (Hrudey, 2009). Many studies have demonstrated that chlorine wash can generate THMs and HAAs in both produce and wash water (Gómez-López, Marín, Medina-Martínez, Gil, & Allende, 2013; Jose Cardador & Gallego, 2012, 2015; López-Gálvez, et al., 2010). Because of such potential toxic effects and concerns, many EU countries have banned the use of chlorine for produce wash (Haute, Sampers, Holvoet, & Uyttendaele, 2013). In the U.S., chlorinated DBPs are tightly regulated by the Environmental Protection Agency (EPA) in drinking water but have not been regulated for produce wash yet (Lee, Huang, & Zhu, 2018). Alternative non-chlorine sanitizers, such as ozone, peracetic acid, hydrogen peroxide, and organic acids, have been studied and proposed to replace chlorine for fresh produce wash (Joshi, Mahendran, Alagusundaram, Norton, & Tiwari, 2013; Meireles, Giaouris, & Simoes, 2016).

Among many different possible alternative sanitizers, activated persulfate shows great potential but unfortunately has not been fully investigated. Persulfate can generate highly reactive free radicals after activation, which can effectively degrade numerous chemical pollutants and has potential in pathogen inactivation (Matzek & Carter, 2016). Its efficacy in microbial reduction has been documented in some research (Garkusheva, et al., 2017; Sabeti, et al., 2017; Wang, Chen, Xie, Shang, & Ma, 2016). Our previous studies have also demonstrated the high efficacy of activated persulfate in eliminating *Escherichia coli* O157:H7 and *Listeria monocytogenes* (Qi, Huang, & Hung, 2018, 2019). Since no chlorine is involved, chlorinated DBPs may be reduced or eliminated. Chu, et al. (2015) even demonstrated UV activated persulfate can effectively degrade DBPs and their precursors. However, if high concentration of halogen ions are present in the activated persulfate solution, toxic DBPs might be generated. For

example, during the degradation process of chlorophenols by Co^{2+} activated persulfate, bromoform and dibromoacetic acid were found in the final products (Wang, et al., 2018).

Another factor to consider for fresh produce wash is the organic quenching effect on the sanitizing chemicals. In the fresh produce industry, one of the convenient practices is to continuously add produce into the washing solutions and recirculate the wash water (Van Haute, et al., 2018). During this recirculation process, organic matter will build up and quench the sanitizing chemicals, such as chlorine (Luo, 2007). Such quenching effects can reduce the concentration of sanitizing chemicals and therefore compromise the pathogen inactivation efficacy. Teng, et al. (2018) indicated the chlorine sanitation effect can be adversely affected when high organic load is present. Organic load can also have the same effect on free radical-based treatment. For example, Yemmireddy and Hung (2015) showed organic matter in food processing water can dramatically decrease the efficacy of UVA activated titanium dioxide in inactivating *E. coli* O157:H7.

Hence, there is a large knowledge gap in whether activated persulfate can be affected by organic load. The objectives of the current study were 1) to determine the effect of organic load from different fresh produce type on the bactericidal efficacy of activated persulfate; 2) to identify the appropriate parameters of organic load to predict the effect on the bactericidal efficacy; 3) to validate the effect of organic load in a simulated produce wash processing.

7.2 Materials and Methods

7.2.1 Chemical and microbial media

Chemical reagents used in the current study were sodium persulfate, ferrous sulfate, sodium hydroxide (10 M), Folin-Ciocalteu's phenol reagent, sodium carbonate, sodium

thiosulfate, sodium chloride, sodium phosphate dibasic, sodium phosphate monobasic monohydrate, Bradford's reagent, sodium pyruvate (SP), and nalidixic acid (NA), methyl tertiary-butyl ether (MTBE), and concentrated sulfuric acid. All chemicals were reagent grade and purchased from Sigma Aldrich (St. Louis, MO, USA) or Fisher Scientific (Fair Lawn, NJ, USA). Microbial media used were tryptic soy agar (TSA), tryptic soy broth (TSB), sorbitol MacConkey agar (SMAC), and DE neutralizing broth. All media were purchased from VWR (Radnor, PA, USA) or Sigma Aldrich (St. Louis, MO, USA).

7.2.2 *E. coli* O157:H7 cocktail preparation

Five strains (1, 4, 5, E009, and 932) of *E. coli* O157:H7 were selected. The cocktail preparation follows the same procedure as described in our previous research (Qi, et al., 2018). Briefly, each strain was activated by transferring a loop of frozen culture (-70 ^{o}C) into TSB and kept in a shaking incubator set at 37 ^{o}C and 120 rpm for 24 h. The incubated broth was further sub-cultured in TSB with 50 mg/L nalidixic acid at 37 ^{o}C and 120 rpm shaking for 24 h. This final broth was used as the stock broth. Working broth was prepared by sub-culturing the stock broth twice, at 37 ^{o}C and 120 rpm shaking for 24 h. Following sub-culture, the broths were centrifuged at 3000×g for 12 min. The cell pellets were washed with 20 mM phosphate buffer saline (PBS) and re-dissolved in PBS. An equal volume of each strain suspension was combined to make the *E. coli* O157:H7 cocktail (about 9.5 log CFU/mL).

7.2.3 Preparation of produce extract

Fresh romaine lettuce, iceberg lettuce, grapes, and strawberries were purchased from a local grocery store and used to make the produce extract. The procedure followed the study of Chen and Hung (2016). Briefly, each produce item was first cut into small pieces and then 50 ± 2 g of cut produce were mixed with 200 mL deionized (DI) water in a Whirl Pak bag. The mixture

was pummeled in a stomacher (Seward, 80 biomaster, Worthing, UK) for 2 min at 260 rpm. The pummeled sample was filtered through a 25 μm filter paper (No. 4 Whatman, Boca Raton, FL, USA) to obtain the produce extract. The chemical oxygen demand (COD) of the produce extract was measured and further dilution of the produce extract was made until the COD level reached around 4500 mg/L.

7.2.4 Treatment

7.2.4.1 Organic load effect in pure solution

A 150-mL glass beaker was used as the treatment reactor. Pre-determined amount of sodium persulfate, sterile DI water, and 1mL *E. coli* O157:H7 cocktail were first added into the beaker. Then the produce extract was added at five different ratios (produce extract to total solution volume ratio): 0:25, 1:25, 3:25, 5:25, and 10:25. The treatment was activated by spiking ferrous sulfate or sodium hydroxide into the beaker. The condition for ferrous activation (PS/Fe^{2+}) was 40 mM persulfate + 13.3 mM ferrous and the condition for alkaline activation (PS/OH^{-}) was 40 mM persulfate + 20 mM sodium hydroxide. The total volume of the treatment solution was controlled at 25 mL. The mixture solution was maintained with a stirring magnetic bar. After 2 min treatment, 1 mL was withdrawn from the beaker and transferred into 9 mL neutralizing buffer (5 g/L sodium thiosulfate and 20 mM PBS). The quenched sample was subject to subsequent microbial analysis.

7.2.4.2 Validation of organic load effect on romaine lettuce

Middle layers of romaine lettuce leaves without obvious physical damage were selected and trimmed to 20 ± 2 g/leaf. Each leaf was spot inoculated with the *E. coli* O157:H7 cocktail on the abaxial side. A small, non-inoculated leaf was used to evenly spread the inoculum over the leaf surface. The inoculated leaves were air dried in a fume hood for 1 h and stored at 4 ^{o}C in a

sealed plastic bag overnight. On the second day, each inoculated leaf was first cut into small pieces using a Nemco 55650 Easy Lettuce Cutter. Then 20 g of chopped leaves were submerged into 800 mL treatment solution in a 2-L glass beaker for 5 min. PS/Fe^{2+} (40 mM), PS/OH^- (40 mM), and NaOCl (100 mg/L and pH 6.5) were the three treatments used. To evaluate the organic load effect, each treatment solution was spiked with the romaine lettuce extract at 5:25 (product extract to total solution volume ratio). The romaine lettuce extract was prepared as described in section 7.2.3. The UV254 value at 5:25 organic load level was controlled at 0.750. The treatment solution was continuously stirred with a magnetic bar. After the treatment, the solution was drained and the washed lettuce leaves were rinsed with DI water followed by placing into 180 mL neutralizing buffer (DE broth, 5 g/L sodium thiosulfate, and 20 mM PBS). The sample was then pummeled for 2 min at 230 rpm and then subject to microbial analysis. In addition, 20 mL of the wash water after each treatment was collected and added to 20 mL double strength TSB with 0.2% SP, 40 mM PBS, and 10 g/L sodium thiosulfate.

7.2.5 Organic load parameter measurement

A total of seven parameters were analyzed. The extract of each produce type was diluted as described in section 7.2.4.1 without addition of the *E. coli* O157:H7 cocktail, persulfate, and persulfate activators (to avoid potential interference). COD and turbidity were analyzed following the Hach method 8000 and Hach method 8237, respectively, with a DR/90 colorimeter (HACH, Loveland, CO, USA). Phenolics, protein, and UV254 were analyzed following the same procedures as described by Chen and Hung (2016). For pH and ORP analysis, the extract was diluted and spiked with 1 mL PBS and activated persulfate as described in section 7.2.4.1. An Accumet meter (Model# AR50, Fisher Scientific, Pittsburgh, PA, USA) was used for pH and ORP measurement.

7.2.6 Microbial analysis

For the pure solution sample (section 7.2.4.1), serial 10-fold dilution was made in PBS and plated on TSA + 0.1% SP (TSASP) plates. For the lettuce sample (section 7.2.4.2), 10-fold diluted samples were plated on both TSA + 0.1% SP + 50mg/L NA (TSASPNA) and SMAC + 0.1%SP + 50mg/L NA (SMACSPNA) plates. The wash water samples (section 7.2.4.2) were incubated at 37 ºC for 24 h and streaked on SMAC plates. All plates were incubated at 37 ºC for 24 h before colony enumeration.

7.2.7 Statistical analysis

The entire experiment was repeated three times. Excel (Micro software, USA) was used for processing means, standard deviations, and correlation coefficients. JMP pro 13 was used for one-way analysis of variance and student t's comparison test. A $P < 0.05$ was considered to be significantly different.

7.3 Results and discussion

7.3.1 Effect of organic load on activated persulfate

The results of organic load effect on *E. coli* O157:H7 reductions are presented in Table 1. Produce extract was diluted at different levels (0:25 to 10:25) to simulate different initial organic loads. COD was used as the primary indicator of organic load and the range was controlled within 2000 mg/L, which is similar to the wash water COD level range in real produce washing practice (Chen & Hung, 2016; Luo, et al., 2018). From the results (Table 1), the effect of organic load on PS/Fe^{2+} was significant. The reductions of *E. coli* O157:H7 decreased from over 8 log CFU/mL to only 1.5 log CFU/mL when the initial organic load was increased from 0:25 to 10:25 for all four produce extracts. The effect was slightly different between produce types. For

romaine lettuce and strawberry, the reductions of *E. coli* O157:H7 showed a sharp decrease (to around 2.2 log CFU/mL reductions) when the organic load was increased to 3:25. Further increase of organic load to 10:25 led to a relatively slight additional inhibition on *E. coli* O157:H7 reductions. For iceberg lettuce and grape, the *E. coli* O157:H7 reductions dropped gradually as the organic load increased from 0:25 to 10:25 (Table 1). This might be associated with different type of organic content in different produce type. For example, the phenolics and UV254 level of romaine lettuce and strawberry were much higher than that of iceberg lettuce and grape at the same COD level. Our previous studies have demonstrated free radicals were the primary compounds of activated persulfate in pathogen inactivation (Qi, et al., 2018, 2019). Mathew, Abraham, and Zakaria (2015) demonstrated that phenolic compounds have great free radical quenching abilities. Kumari and Gupta (2018) indicated UV254 absorbance can be used to indicate the natural organic matter and aromatic compound (e.g. phenolic compound) level in water solution. Therefore, with a much higher level of phenolics and UV254 value in romaine lettuce and strawberry extract, a much lower microbial reduction should be expected due to the radical quenching effect.

On the other hand, the effect of organic load on PS/OH^- treatment was not as significant as PS/Fe^{2+}. Compared to the control treatment without organic load spiking, the *E. coli* O157:H7 reductions at the highest spiked organic load level dropped about 2 to 3 log CFU/mL (Table 1). Iceberg lettuce extract showed the minimum effect in that the reductions only reduced from 5.81 to 4.13 log CFU/mL. The drop in reductions can be explained by increased organic load as well as the pH effect. As the organic load increased, the pH of PS/OH^- treatment solution also decreased slightly (Table 1). It has been demonstrated that alkalinity is an important factor for persulfate activation (Qi, et al., 2018). Therefore, the decrease of pH can cause suppressed

persulfate activation and lower the free radical concentrations, leading to reduced microbial reductions. On the other hand, the different susceptibility towards organic load effect between PS/Fe^{2+} and PS/OH^- can be explained by the primary type of radical generated between the two activation methods. Superoxide radical is the primary compound of PS/OH^- in pathogen inactivation while hydroxyl radical is the primary compound of PS/Fe^{2+} in pathogen inactivation (Qi, et al., 2018). Phenolic compounds were demonstrated to have a higher quenching effect against hydroxyl radical than superoxide radical (Mathew, et al., 2015). Therefore, at the same organic load level, more hydroxyl radical of PS/Fe^{2+} will be neutralized than superoxide radical of PS/OH^-, leading to a higher organic load effect on microbial inactivation.

The effect of organic load on free radicals was also observed in other research studies. Yemmireddy and Hung (2015) studied the effect of UV light activated titanium dioxide in inactivating *E. coli* O157:H7 in presence of produce wash water with different organic load levels. They demonstrated the pathogen inactivation efficacy was gradually decreased by increasing the produce wash water content from 0 to 100%. Similar effects of organic load were also observed for UVA light activated gallic acid treatment against *E. coli* O157:H7 by Cossu, et al. (2016) where COD at 2000 mg/L exhibited a strong inhibition effect on the pathogen inactivation (gallic acid < 5 mM). Chlorine-based sanitizers, such as chlorine dioxide, were also found to be sensitive towards organic load effects. Banach, et al. (2017) observed over 2 log CFU/mL decrease of *Salmonella* Typhimurium and *E. coli* reductions when the total organic carbon in ClO_2 solution was increased from 177 to 354 mg/L. The organic load effect on free radical-based treatment was only extensively evaluated for chemical degradations. Acero, Benítez, Real, and Rodríguez (2018) found that the degradation efficacy of emerging contaminant chemicals by UV activated persulfate can be easily inhibited by humic acid. The

strong quenching effect of humic acid on sulfate and hydroxyl radicals was considered as the primary reason. Both Zhou, Ferronato, Chovelon, Sleiman, and Richard (2017) and Ji, et al. (2017) also demonstrated natural organic matter can strongly reduce the efficacy of photo activated persulfate in degrading chemical contaminants, such as diatrizoate and antibiotic compounds. The high radical quenching and reducing power of natural organic matter was deemed to be the major inhibition source.

7.3.2 Parameters for organic load effect indicator

To investigate which organic load parameter can be used to indicate the effect on pathogen inactivation, the correlation coefficients between *E. coli* O157:H7 reductions against each organic load parameter per produce type per activation method were calculated. The results are shown in Table 2. COD, phenolics, and UV254 showed the best correlation coefficient values with the microbial reductions, with the coefficients of more than 0.78 per produce type per activation treatment. Protein showed comparable coefficient values for romaine lettuce, iceberg lettuce, and strawberry, but poor values for grape (0.78 and 0.67). Turbidity's coefficient values were high. However, since many other factors can contribute to turbidity, such as dirt, turbidity may not be a reliable indicator to predict the organic load effect. Furthermore, pH and ORP did not demonstrate consistent correlation coefficient values among the produce and therefore were not considered as organic load effect indicators. Based on the discussion above, it was concluded that COD, phenolics, and UV254 can be used as reliable indicators to predict the organic load effect on the pathogen inactivation efficacy of PS/Fe^{2+} and PS/OH^{-}.

Plots of *E. coli* O157:H7 reductions against COD, phenolics, and UV254 were made to further investigate their relationships. As figure 1 shows, the effect of these three parameters were different for each activation method. For PS/Fe^{2+}, the microbial reduction changing trends

were close to logarithmic. That means by increasing a small level of COD, phenolics, or UV254 a dramatic decrease of microbial reductions will be observed while further increase of these parameter levels will not significantly affect the reductions. On the other hand, for PS/OH^-, the reduction changing trends were more linear. That means by increasing COD, phenolics, or UV254 level, the microbial reductions will gradually decrease. Also, at the same COD, phenolics, or UV254 level, the effect can be different for each produce. For example, by increasing the UV254 level, the *E. coli* O157:H7 reductions in presence of grape extract underwent a sharper drop compared with the other three produce types (Figure 1). Although COD, phenolics, and UV254 all showed high correlation coefficients with microbial reductions, UV254 was selected as the most appropriate parameter to indicate the organic load effect. This is due to the consideration of time needed for each measurement. Both COD and phenolics analysis need about 2 h for sample preparation and digestion, which may not be applicable for on-line sensing during produce washing. UV254, on the other hand, can be measured in 30 s since the sample preparation only involved a single-step membrane filtration and the absorbance reading can be easily measured by a spectrophotometer.

7.3.3 Organic load effect on romaine lettuce

The above observations were from produce extract to simulate different organic load level in aqueous solution. To further investigate the organic load in real situation, romaine lettuce was used to validate the organic load effect for real produce because it is a commonly consumed fresh produce item and has been linked to recent *E. coli* O157:H7 outbreaks (CDC). The two activated persulfate treatments at the same condition as described in section 7.3.2 and NaOCl at 100 mg/L with or without the addition of romaine lettuce extract were used to wash *E. coli* O157:H7 inoculated romaine lettuce leaves. Organic load spiked into the washing solution was

controlled at UV254 of 0.750 because this UV254 level showed a high inhibition effect on activated persulfate (Table 1). All treated samples were plated on two types of agar plates, TSASPNA (less selective) and SMACSPNA (more selective), to get more reliable plate counts. From the results, the reduction numbers from SMACSPNA were a little bit higher than that of TSASPNA. This might be because some pathogen cells were injured during the sanitizing treatment and injured cells are not able to recover on selective agar plates. However, the differences were generally minor (less than 0.2 log CFU/g) and therefore the average reduction numbers were used to represent *E. coli* O157:H7 reductions.

As Table 3 shows, 2.38 to 2.99 log CFU/g reductions were achieved. Treatments without organic load all showed higher reductions than the respective treatment with organic load. However, only the differences for PS/Fe^{2+} were significant ($P < 0.05$) (2.99 vs 2.33 log CFU/g). This result corresponds to the results from Table 1 that PS/Fe^{2+} was more sensitive to the organic load effect than PS/OH^-. For NaOCl, the addition of organic load will react with free chlorine (Chen & Hung, 2016). The organic load effect on NaOCl reported here is similar to the results of Chen and Hung (2018) that the decrease of free chlorine content from 90 to 10 mg/L in NaOCl due to organic load quenching effect will not cause significant drop of *E. coli* O157:H7 reductions on iceberg lettuce. In addition, the wash water after each treatment was collected and enriched to detect the survival of *E. coli* O157:H7 and the potential for cross-contamination. When no additional organic load was added, all three treatments showed complete (0/3) or near complete (1/3) negative enrichment results. However, when organic load was added, both PS/Fe^{2+} and PS/OH^- showed complete positive (3/3) enrichment results while NaOCl still showed near complete negative (1/3) enrichment results. The effect of organic load on NaOCl was minimum because there were still about 35 mg/L residual free chlorine (data not shown)

after the organic load spiking and 5 min reaction. The residual free chlorine concentration was high enough to continuously inactivate pathogen cells. For activated persulfate, however, majority of the free radicals were quenched by organic matters and therefore activated persulfate were less effective to inactivate foodborne pathogens.

7.4 Conclusions

The current study demonstrated organic load from fresh produce will have a significant inhibition effect on the *E. coli* O157:H7 inactivation efficacy of activated persulfate. By increasing the organic load level, the pathogen inactivation efficacy of activated persulfate will be dramatically suppressed. PS/Fe^{2+} is more susceptible to the organic load effect than PS/OH^{-}. This organic load effect can be indicated by COD, phenolics, and UV254 absorbance. However, UV254 was considered as a more appropriate indicator for the organic load effect due to easiness of measurement. Such organic load effect was also successfully validated on romaine lettuce. A lower reduction of *E. coli* O157:H7 was found for both activated persulfate treatment and sodium hypochlorite. The build-up of organic load in wash water can also increase the potential of pathogen cross-contamination when PS/Fe^{2+} and PS/OH^{-} are used as sanitizers. Therefore, if activated persulfate was used for produce washing in a real situation, the organic load in wash water must be carefully monitored to maintain the high efficacy in pathogen inactivation.

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Table 7.1. Reductions of *E. coli* O157:H7 populations and wash water parameters of each produce at different organic load.

							PS/Fe^{2+}			PS/OH^-		
	Organic load	COD (mg/L)	Phenolics (mg/L)	Protein (mg/L)	UV254	Turbidity (FAU)	pH	ORP (mV)	Reduction (log CFU/mL)	pH	ORP (mV)	Reduction (log CFU/mL)
Romaine	0:25	0.0	0.0	0.0	0.000	0.0	2.58	642.7	8.11	12.45	230.83	5.88
lettuce	1:25	153.0	1.03	11.91	0.136	13.7	2.58	646.53	6.46	12.45	211.47	5.01
	3:25	526.3	4.00	38.84	0.420	39.3	2.57	638.57	2.32	12.44	183.83	4.33
	5:25	949.3	9.14	64.74	0.728	68.7	2.57	628.80	1.89	12.39	161.33	4.08
	10:25	1847.3	16.68	122.26	1.472	134.0	2.57	616.67	1.71	12.35	130.17	3.70
Iceberg	0:25	0.0	0.00	0.00	0.000	0.0	2.58	647.40	8.16	12.43	123.90	5.81
lettuce	1:25	146.7	0.12	2.82	0.044	3.3	2.58	646.03	6.80	12.43	125.73	5.60
	3:25	434.3	1.27	9.77	0.131	10.7	2.57	636.07	4.20	12.40	116.50	4.72
	5:25	865.3	2.34	18.51	0.225	17.7	2.56	631.97	2.78	12.36	99.43	4.49
	10:25	1695.3	5.42	39.45	0.452	38.0	2.55	627.40	2.11	12.31	94.17	4.13
Grape	0:25	0.0	0.00	0.00	0.000	0.0	2.59	675.97	8.08	12.44	136.93	6.43
	1:25	191.0	0.38	2.29	0.011	0.3	2.58	472.37	7.42	12.43	134.77	6.37
	3:25	553.0	0.69	3.44	0.029	2.0	2.57	665.87	6.55	12.41	119.27	6.09
	5:25	908.0	1.98	4.27	0.049	3.3	2.57	652.67	3.50	12.35	91.07	5.34
	10:25	1880.0	4.78	4.90	0.098	7.7	2.55	641.10	1.76	12.30	62.00	3.67
Strawberry	0:25	0.0	0.00	0.00	0.000	0.0	2.58	674.40	8.03	12.45	220.43	5.88
	1:25	172.3	2.70	1.73	0.131	4.0	2.58	672.37	5.84	12.45	188.90	5.84
	3:25	530.3	9.36	3.85	0.381	13.0	2.56	655.57	2.24	12.41	127.20	4.81
	5:25	983.0	16.75	6.78	0.643	20.0	2.54	643.70	1.71	12.40	96.43	3.79
	10:25	1882.3	34.22	11.63	1.270	44.3	2.54	630.33	1.30	12.35	32.03	2.22

The initial *E. coli* O157:H7 population was 8.43 ± 0.05 log CFU/mL. PS/Fe^{2+} was at 40 mM persulfate + 13.3 mM ferrous and PS/OH^- was at 40 mM persulfate + 20 mM NaOH. All treatments were conducted for 2 min.

Table 7.2. Correlation coefficients between reductions of *E. coli* O157:H7 populations and different wash water parameters.

Treatment		COD	Phenolics	Protein	UV254	Turbidity	pH	ORP
PS/Fe^{2+}	Romaine lettuce	-0.80	-0.78	-0.80	-0.77	-0.78	0.49	0.60
	Iceberg lettuce	-0.88	-0.85	-0.87	-0.88	-0.87	0.60	0.90
	Grape	-0.96	-0.94	-0.78	-0.96	-0.89	0.68	-0.20
	Strawberry	-0.81	-0.80	-0.83	-0.82	-0.79	0.87	0.89
PS/OH^-	Romaine lettuce	-0.86	-0.82	-0.83	-0.81	-0.81	0.81	0.87
	Iceberg lettuce	-0.88	-0.88	-0.89	-0.90	-0.87	0.67	0.82
	Grape	-0.98	-0.99	-0.67	-0.98	-0.90	0.96	0.91
	Strawberry	-0.97	-0.97	-0.92	-0.97	-0.95	0.94	0.94

PS/Fe^{2+} was at 40 mM persulfate + 13.3 mM ferrous and PS/OH^- was at 40 mM persulfate + 20 mM NaOH.

Table 7.3. Reductions of *E. coli* O157:H7 populations on romaine lettuce after different sanitizer treatment with or without organic load.

	Reduction (log CFU/g)			Wash
	TSASPNA	SMACSPNA	Average[a]	Water[b]
PS/Fe^{2+}	2.97 ± 0.29	3.01 ± 0.25	2.99 ± 0.26[A]	0/3
PS/Fe^{2+} + organic	2.22 ± 0.29	2.43 ± 0.11	2.33 ± 0.19[B]	3/3
PS/OH^-	2.51 ± 0.11	2.60 ± 0.21	2.56 ± 0.15[B]	1/3
PS/OH^- + organic	2.35 ± 0.09	2.53 ± 0.10	2.44 ± 0.07[B]	3/3
NaOCl	2.58 ± 0.37	2.76 ± 0.44	2.67 ± 0.40[AB]	1/3
NaOCl + organic	2.28 ± 0.07	2.47 ± 0.05	2.38 ± 0.05[B]	1/3

[a]Values followed by the same capital letter are not significantly different ($P \geq 0.05$).

[b]n = 0/3 means out of 3 trials, no *E. coli* O157:H7 was found after enrichment.

PS/Fe^{2+} was at 40 mM persulfate + 13.3 mM ferrous, PS/OH^- was at 40 mM persulfate + 20 mM NaOH, and NaOCl was at 100 mg/L and pH 6.50. The organic load level spiked in each treatment was at UV254 of 0.750.

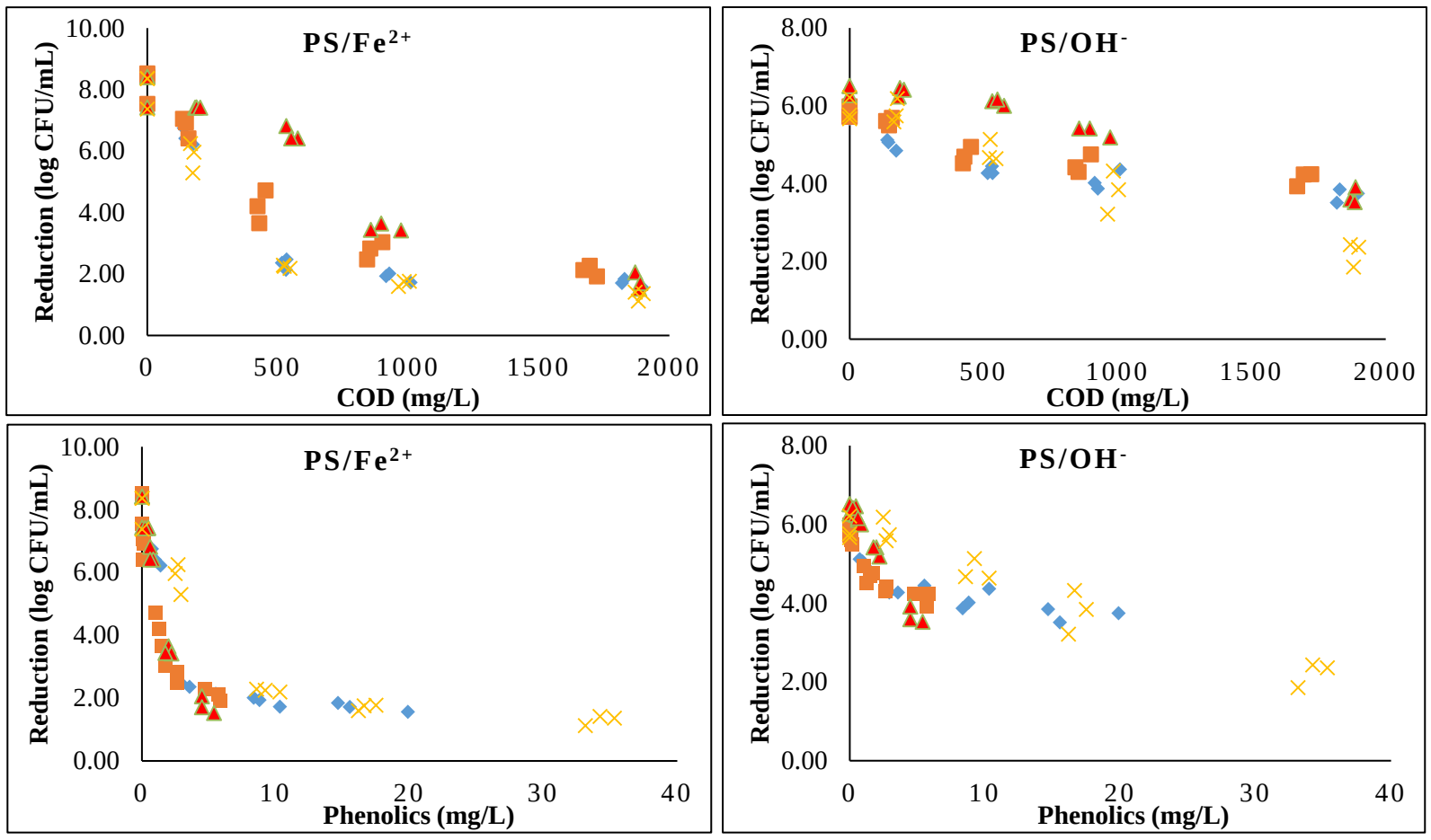

Figure 7.1. Plot of *E. coli* O157:H7 population reductions against COD, phenolic content, and UV254 of romaine lettuce (◆), iceberg lettuce (■), grape (▲), and strawberry (×) after treatment of PS/Fe^{2+} and PS/OH^{-}.

CHAPTER 8

SUMMARY AND CONCLUSIONS

This research systematically evaluated the efficacy of ferrous and alkaline activated persulfate in inactivating *E. coli* O157:H7 and *L. monocytogenes* and the potential of using activated persulfate for fresh produce sanitation. The main hypothesis was: Activated persulfate solution is capable of inactivating and removing foodborne pathogens from fresh produce surface, and the efficacy is dependent on the activator, activator to persulfate ratios, the type of pathogen, and the organic load. Four research objectives were established to test the main hypothesis and five research chapters were conducted.

The first two research chapters evaluated the efficacy of ferrous and alkaline activated persulfate in inactivating foodborne pathogens in pure solution. The results indicated that the efficacy was persulfate to activator ratio dependent. For ferrous activation, persulfate to ferrous molar ratio of 3:1 showed the highest microbial inactivation efficacy. For alkaline activation, higher alkalinity improved the inactivation efficacy. For both activation methods, higher initial persulfate concentration led to higher microbial reductions. Free radicals were demonstrated to be the major compounds in pathogen inactivation and they differed between each activation method. For ferrous activation, hydroxyl radical was the primary radical in pathogen inactivation, while for alkaline activation, superoxide radical was the primary radical in pathogen inactivation. It was also demonstrated the efficacy of the two activation methods can last for several hours.

The third research chapter deduced the possible inactivation mechanism of pathogen cell when treated by ferrous and alkaline activated persulfate. Significant cell injury of both *E. coli* O157:H7 and *L. monocytogenes* were found after activated persulfate treatment. Intracellular leakages of protein and DNA were observed. The dehydrogenase activity of the two pathogens were also inhibited after the activated persulfate treatment. Further TEM and SEM imaging revealed that there were significant damage on cell surface structures as well as cytoplasm leakages and agglutination. Therefore, based on the results, the primary inactivation mechanism was considered as: activated persulfate treatment can cause significant damage on cell envelope structures, significant leakage of intracellular materials, and key enzyme inhibition, which finally lead to cell death.

The fourth and fifth research chapters evaluated the potential of activated persulfate for fresh produce sanitation. It was demonstrated up to 3.5 log CFU/g reductions can be achieved using ferrous and alkaline activated persulfate on romaine lettuce inoculated with *E. coli* O157:H7 or *L. monocytogenes*. The efficacy was dependent on the initial persulfate level and treatment time. The results also demonstrated that, at appropriate conditions, cross-contamination can be effectively prevented. The efficacy observed with activated persulfate were comparable with chlorine sanitizers and peracetic acid. On the other hand, activated persulfate was found to be sensitive to organic load. The pathogen reduction efficacy can be significantly reduced in the presence of organic matter. COD, phenolics, and UV254 absorbance were found to be reliable indicators for the organic load effect.

The overall findings of this project provide the produce industry with an alternative sanitizer that can be used to replace the traditional chlorine-based sanitizers. With activated

persulfate, chlorinated DBPs can be reduced or eliminated, and thus the food safety and consumers' health can be ensured.