CHARACTERIZATION OF ZINC OXIDE NANOPARTICLES AND THEIR APPLICATIONS IN FOOD SAFETY

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By

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CHARACTERIZATION OF ZINC OXIDE NANOPARTICLES AND THEIR APPLICATIONS IN FOOD SAFETY

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A candidate for the degree of Master of Science

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DEDICATION

To mom, dad and the living memory of grandma

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CHARACTERIZATION OF ZINC OXIDE NANOPARTICLES AND THEIR APPLICATIONS IN FOOD SAFETY

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ABSTRACT

In this study, ZnO NPs suspension was studied for their antibacterial activities against *Escherichia coli* O157:H7. Beef cuts inoculated with *E. coli* O157:H7 were wrapped with 5% (wt %) yam starch films incorporated with ZnO NPs suspension of 0, 6 and 12 mM and refrigerated at 4°C. An average of 0.5-log reduction of the bacteria growth of the cocktail mixture of three *E. coli* O157:H7 strains was observed with yam starch films containing 6 or 12 mM ZnO NPs in three replications after 8 days.

Various concentrations of ZnO NPs powder from 0.05 to 1% w/w were added to corn starch. The presence and characterization of ZnO NPs in corn starch was investigated by scanning electron microscopy (SEM), and transmission electron microscopy (TEM), and energy-dispersive X-ray spectroscopy (EDS). Quantification of ZnO NPs was determined by inductively coupled plasma optical emission spectrometry (ICP-OES).

To the best of our knowledge, this is the first systematic methodologies presented for detection, characterization, and quantification of ENPs in a food sample by a combination of methods. It could potentially be applied to other common NPs such as TiO_2 NPs and MgO NPs in other food products.

CHAPTER 1

INTRODUCTION

1.1 Need for the research

One of the leading causes of many foodborne outbreaks in the United States over the years is *Escherichia coli* O157:H7 (Al-Qadiri and others 2006). It is the most important enterohemorrhagic *E. coli* serotypes that can lead to inflammation of the colon and abdominal pain with bloody stools and cause bloody diarrhea in some cases (Al-Qadiri and others 2006). The infective dose is as low as 10 cells (Jay 2000). Fever and vomiting is rare but could also occur. *E. coli* O157:H7 has been implicated in many foods, including lettuce, radish sprouts, alfalfa, apple cider, raw milk, ground beef, and roast beef (Hathcox and Beuchat 1996; Keene and others 1997; Smith and Fratamico 2005). In 2006, over 200 people in 26 states were infected with *E. coli* O157:H7 from consuming contaminated spinach, and three deaths were reported (Gelting and others 2011).

Consequently, numerous works has been conducted in recent years on developing innovative packaging materials that can inhibit the growth of *E. coli* O157:H7 for food products (Sun and Sun 2001; Jin and Zhang 2008; Hong and others 2009). Some promising materials have been suggested, such as films and coatings formulated with polysaccharides, proteins and lipids. Among them, starch films, as decent oxygen and carbon dioxide barriers, have been proven to possess some potential (Mali and Grossmann 2003). Being biodegradable, the use of starch films can also help reduce the amount of plastic waste.

In addition to starch films, antibacterial agents can be added to enhance the antibacterial effect. Inorganic materials such as metal oxides have been previously studied to show impressive antibacterial activity against *E. coli* O157:H7 (Sawai and others 1998; Jia H 2008). They are particularly favored due to the ability to endure harsh processing conditions compared with organic substances (Stoimenov and others 2002). ZnO has been widely used as a zinc fortifier in human diet and is listed by the FDA as "Generally Recognized as Safe (GRAS)". The adoption of ZnO nanoparticles (NPs) has attracted the attention of food microbiologists in recent years. Both ZnO dry powders and suspensions have been studied and proved to exhibit antibacterial ability at certain concentrations. Previous research suggested that ZnO NPs are effective for inhibiting the growth of both Gram-positive and Gram-negative bacteria (1995a; Sawai and others 1995b; 1996a; 1996b) and the antibacterial ability is believed to rely mainly on their large surface area and concentrations rather than the crystalline structure and particle shape (Yamamoto and others 1998).

With the brisk developments of nanotechnology in recent years, the applications of nanostructures and nanomaterials have gone beyond research stages into the commercial marketplace (Wardak and others 2008). The majority of nanomaterial-containing products are related to cosmetics, clothing, and computer chips (Benn and Westerhoff 2008; Wokovich and others 2009; Marambio-Jones and Hoek 2010). Needless to say, the prospect of nanotechnology in the food industry is immense and food products containing nanomaterials will inevitably be growing in the near future.

Drawbacks of NPs remains, however, as safety issues with NPs such as ZnO NPs have been brought to the attention of both scientists and the general public (Service 2003; Oberdörster and others 2005). Consequently, there is a growing concern in recent years from consumers regarding intentional or unintentional contamination of foods by engineered nanoparticles (ENPs) from packaging materials, nano-sized pesticides, and other sources. ENPs are considered more active than their macromolecule counterparts due to a high surface area per mass (Oberdörster 2005). Studies have also shown that ï ï ï ï 3苕:萑□搒ð□葞:葠□摧瘲□□r, translocate to other organs, and impair DNA other organs, and impair DNA (Albrecht and others 2006). Generally, the risks of ENPs are measured by their toxicity and the extent of them coming into contact with other organisms (Mueller and Nowack 2008). Unfortunately, few studies to date have addressed a sound approach to quantify and characterize trace ENPs.

1.2 Objectives of the study

In light of the issues discussed above, our study was devoted to constructing a novel antimicrobial packaging material with the help of plasticized yam starch films and ZnO NPs suspension. Top sirloin beef was chosen as the packed product and *Escherichia coli* O157:H7 was selected as the target microorganism. In addition, we aimed to develop a combination of methods that are sensitive and accurate enough to detect, characterize, and quantify low concentrations of ENPs in food products. Since no single technique is sufficient to handle the task, a combination of technologies were investigated, including scanning electron microscopy (SEM), transmission electron

microscopy (TEM), energy dispersive spectrometer (EDS), and inductively coupled plasma optical emission spectrometry (ICP-OES).

CHAPTER 2

REVIEW OF LITERATURE

2.1 The antibacterial activity of ZnO NPs

Antibacterial agents are of great importance to numerous industries, such as environmental, food, packaging, medical, healthcare, decoration industries, and so forth. They are generally classified into two types: organic or inorganic agents (Zhang and others 2007). As aforementioned, the advantages of inorganic antibacterial agents over organic antibacterial agents include higher stability and improved safety, especially under high pressure and/or temperature (Sawai 2003). These attributes have attracted much interest for the control of microorganisms, particularly pathogens. A number of ceramic powders have been evaluated and proven to exhibit antibacterial activities, such as TiO_2, CaO, MgO, and ZnO (Sawai and others 2000; Yamamoto and others 2000; Gogniat and others 2006), with ZnO being one of the most studied. In 2006, a preliminary study of the antibacterial effect of ZnO NPs against *Escherichia coli* was reported by Brayner and others (2006). The results showed that at a concentration of 1.3 mM or lower, the growth of *E. coli* was not significantly affected. In fact, at 1.0 and 1.3 mM, more *E. coli* colonies than in the control group was observed, suggesting that ZnO NPs are not inhibitory or toxic against *E. coli* at these levels. However, as the concentration of ZnO NPs increased, fewer colonies were recorded. At 3.0 to 10 mM, ZnO NPs caused 100% inhibition of bacterial growth.

According to another study by Jin and others (2009), ZnO quantum dots, and nanoparticles of purified powdered ZnO, were effective in reducing the cell population of *Listeria monocytogenes, Salmonella* Enteritidis, and *E. coli* O157:H7. The antimicrobial efficacy was concentration-dependent, as higher ZnO concentration resulted in a greater reduction of growth. To be specific, 3.2 mg ZnO/mL treatment caused a 5.3 log reduction of *L. monocytogenes* and a 6.0 log reduction of *E. coli* O157:H7 in growth media after 2-d incubation. ZnO at 1.12 and 0.28 mg/mL concentrations were investigated against *Salmonella* for antibacterial effects and as a consequence, cell growth was reduced by 6.1 and 4.1 log CFU/mL, respectively. Despite the fact that the ZnO levels applied in this study were considerably higher than those used in most researches, the results demonstrate the antibacterial activity of ZnO NPs over a spectrum of bacteria. This was further supported by a study conducted by Jones and others (2008), where ZnO NPs were tested for their inhibitory effect on various bacteria, including *Staphylocoocus aureus*, *S. epidermidis*, *S. pyogenes* and *Bacillus subtilis*. The growth reduction was greater at higher ZnO concentrations and/or smaller particle size, suggesting that the antibacterial mechanism of ZnO nanoparticles against bacteria cells was through accumulation of ZnO NPs inside the bacterial cell membrane.

Moreover, lower concentrations were used in a study by Liu and others (2009) where ZnO NP suspensions were analyzed at concentrations of 3, 6, and 12 mM specifically against *E. coli* O157:H7. Results showed that tryptic soy agar (TSA) plates with 3 and 6 mM ZnO nanoparticles exhibited less bacterial growth over that of a

control while at 12 mM, the growth of *E. coli* O157:H7 was completely inhibited. The researcher also suggested a mode of action of ZnO NPs, which was similar to that by Jones (2008) that ZnO NPs damaged the bacterial cell membrane and caused a leakage of intracellular contents.

More studies in recent years have been reported (Sawai and Yoshikawa 2004; Ghule and others 2006; Reddy and others 2007; Li and others 2008; Xie and others 2011) regarding the antimicrobial effect of ZnO nanoparticles and their inhibitory mechanisms of bacteria and fungi. Although no definitive conclusion on the mechanism of action of ZnO NPs against bacteria has been drawn, it is well recognized that the higher the concentration and the smaller the particle size, the greater the inhibitory effect is.

2.2 The toxicity of ZnO NPs

Engineered NPs have exhibited exquisite physiochemical properties on which the medical, environmental, and even food industries have begun to capitalize by manufacturing ZnO NPs-containing products such as sunscreens, toothpastes, inks, clothing, and food products (Hussain and others 2005). Nanoparticle-based electrochemical DNA sensors that are ultrasensitive in detecting selected DNA sequence or mutated genes have also been proposed and are currently under development (Tien-Li and others 2005; Chen and others 2007).

Unfortunately, the expanding applications of nanomaterials have posed serious concerns for their impact on both the environment and human health. A workshop supported by the National Science Foundation (NSF) and the U.S. Environmental Health

Agency (EPA) has recognized several critical risk assessment issues that engineered nanomaterials have brought up, including exposure assessment, toxicology, ability to extrapolate, environmental and biological fates, transport, persistence, transformation, and overall sustainability (Jeng and Swanson 2006).

As for ZnO NPs, their antimicrobial effect over a wide spectrum of microorganisms is advantageous for promoting food safety but can be detrimental to the ecosystem as well when contaminating foods or ground waters (Adams and others 2006). The ultra-small size that endows them with exceptional antimicrobial activity also renders them more active than their bulk counterparts and allows them to be easier to spread into unexpected and unwanted locations. Since their behaviors have not been fully understood, it could raise a big concern when they unintentionally cross-contaminate in food products and thus could threaten human health.

Studies have been carried out to examine the toxicity of ZnO NPs and other metal oxide NPs to mammalian cells and organs (Jeng and Swanson 2006; Lai and others 2008; Wang and others 2008; Liu and others 2009), and ZnO NPs were found to cause a more severe damage than other metal oxide nanoparticles in many cases. In the investigation by Jeng and Swanson (2006), results showed that neuro-2A (mouse neuroblastoma, CCL-131, ATCC), when exposed to ZnO nanoparticles of 100 µg/mL or more, became abnormal in size, and displayed cellular shrinkage. At 50 to 100 µg/mL, ZnO nanoparticles caused a substantial decrease of mitochondrial function. Lactate

dehydrogenase leakage and apoptosis were also observed in cells exposed to ZnO nanoparticles.

Animal studies on the toxicity of ZnO NPs were conducted too, attracting public concerns. According to (Wang and others 2008), 20- and 120- nm ZnO nanoparticles were mainly discovered in the bone, kidney and pancreas in healthy adult mice after administration. Low and median doses of 20-nm ZnO nanoparticles might induce blood viscosity. Moreover, experimental mice had dose-effect pathological damages in stomach, liver, heart and spleen when treated with 120-nm ZnO. Consequently, they suggested that the liver, spleen, heart, pancreas and bone are the target organs for 20- and 120- nm ZnO oral exposure. The findings provided valuable references for studies investigating the toxicity of ZnO nanoparticles to human health when digested orally. Researches on the impact of ZnO nanoparticles on human cells were followed. (Lin and others 2009) investigated the toxicity of both micro- and nano-sized ZnO particles in human lung epithelial cells. Results demonstrated that exposure to both sizes of ZnO particles could lead to cytotoxicity that was dose- and time- dependent. Oxidative stress, lipid peroxidation, cell membrane damage, and oxidative DNA damage were observed. When compared with other metal oxides, ZnO particles also exhibited a steeper dose-response pattern.

More evidence was supported, as seen in a study by Lai and others (2008), where ZnO NPs were determined to be the most effective out of three metal oxide nanoparticles in inducing cell death in human astrocytoma U87 cells. However, ZnO

nanoparticles showed no significant effect on lowering the survival of primary human T cells at concentrations of 5 mM or lower. Further investigation is needed as the researcher provided no explanation to this phenomenon.

Nonetheless, it is undeniable that ZnO NPs possess potential risk to both the environment and human health, although scientists are continuing looking into the mechanism underlying the toxic effect. Most evidence presented to date suggests a theory that the toxic impact is dose-, size- and exposure time-dependent, in that the higher the dose, the smaller the size, and the longer the exposure time, thus the more detrimental the ZnO NPs are.

Workers who occupationally handle nanoparticles should conduct their professions with extreme caution and discretion while the public should be informed of the risks of exposing themselves to nanoparticles and be made aware of the nanomaterial-containing products around them.

2.3 Detection and characterization of trace amount of nanoparticles

With the toxicity of nanoparticles being widely recognized and the well-known fact that the ingestion of metallic particulate matters can have adverse effects to human bodies (Gatti and others 2009), it comes a critical task of detecting their presence and ultimately characterizing the detected nanosized matters. Nanoparticles have been extensively used to detect target DNA or genes, locate tumor and cancer cells, and improve drug delivery (Jeng and Swanson 2006). Contradictorily, limited research has addressed the issue of detecting nanoparticles when contamination occurs or residues

are left in treatment tanks. Moreover, with nanoparticles spanning a wide range of size, distribution and structure, developing techniques that are easily accessible to characterize them becomes a tough job (Weinberg and others 2011). To make it more difficult, the amount of nanoparticle contamination is generally low, due to the limited amount manufacturers put into their products or facilities.

Analyzing engineered nanoparticles (ENPs) in waters and distinguishing them from natural nanoparticles have been discussed over the years. Very recently, Zheng and others (2011) outlined a protocol of combining energy dispersive X-ray (EDX) microanalysis with traditional TEM technique to fulfill the task of identifying nanoparticles in tissue or cultured cell thin sections. The same principles can be applied to identify nanoparticles in powdered matter. However, as promising as it appears, there is always room for improvement, one crucial aspect is to quantify nanoparticles pollutants rather than merely identify them.

2.4 Yam starch films

Many commercial foods that are nutritious and moist suffer from quality and economical loss during storage due to changes of physical and chemical properties. Moreover, microbial spoilage commonly occurs on the food surface. Traditional solutions involve adding antimicrobial agents to the foods, but they could disturb many substances and nutrients in the food and their antimicrobial activity could be inhibited (Durango and others 2006). Developing safe, low-cost, antimicrobial and environmental-friendly packaging materials thus becomes crucial to the food industry.

Starch-based films and coatings have shown promises in meeting these challenges (García and others 2000). Starches are cheap, abundant, biodegradable, renewable, and edible. Under certain conditions of relative humidity and temperatures, starch films have proven to be a good barrier to oxygen and carbon dioxide, but not so efficient to water vapor (Mali and Grossmann 2003). They can therefore function by lowering the respiration rate of the packaged product and providing a disadvantageous environment for deteriorating and pathogenic microorganisms and consequently inhibiting their growth. Their application to vegetables and fruits such as tomato, cucumber, peppers, carrots, strawberries, and apples have been extensively studied (Drake and others 1987; El Ghaouth and others 1991). Among them, chitosan-based films and coatings have had much success in extending shelf life and preserving the surface color of various products.

Other materials such as yam starch have been brought to the attention of researchers. One of the favorable attributes yam starch possesses is its high amylose content. Amylose plays a significant role in the film forming capacity of starches (Mali and others 2002). Yam starch contains approximately 30% amylose and thus, is an excellent candidate for film production. Another key component for film formation is plasticizers, which must be compatible with the starch polymer. It is essential because films formed solely by starches are brittle and fragile. With the addition of plasticizer, films become more flexible and extensible. Some of the most adopted plasticizers in film formulations are hydrophilic compounds including glycerol, sorbitol, and polyethylene glycol (Gontard and others 1993).

The mechanical properties and antimicrobial effect of yam starch films have been explored. In one study by Mali and Grossmann (2003), yam starch films formed with 4.00 g/100 g yam starch and 1.30 or 2.00 g/100 g glycerol of were efficient in limiting decay of fresh strawberries (*Fragaria ananassa*). The shelf life of the fruits was extended from 14 days of the control to 21 days regarding the microbiological counts. Although different mechanical properties were observed as glycerol content varied, no difference was noticed as a function of antimicrobial activity. This suggests the antimicrobial effect of the films were determined by the yam starch itself.

In another study (Durango and others 2006), chitosan was added to strengthen the beneficial properties of yam starch films. Results showed that chitosan-added yam starch was effective at controlling the microbiota artificially spiked in minimally processed carrots, as the growth of lactic acid bacteria, total coliforms, psychrotrophs, yeast and molds and mesophilic aerobes was all inhibited to some extent. Although it was worth mentioning that according to Durango's results, yam starch coating without chitosan, in fact, facilitated the growth of mesophilic and psychrotrophic bacteria. One explanation to this phenomenon is that the aforementioned microorganisms have captivated yam starch as a source of energy. This suggests that when dealing with mesophilic and psychrotrophic bacteria, yam starch alone is not sufficient; additional antimicrobial agents might be needed.

Barrier, mechanical and optical characteristics of yam starch films with plasticizer were further studied (Mali and others 2004). Glycerol content increased water vapor

and O_2 permeability, as well as puncture deformation. Films were more opaque as a function of film thickness. Of all combinations of glycerol and yam starch, films with 4.0% w/w starch, 1.30% w/w glycerol and 0.11 mm thickness resulted in the highest puncture strength.

CHAPTER 3

MATERIALS AND METHODS

3.1 ZnO NPs, food sample and bacterial strains

ZnO NP powders were purchased from Nanostructured & Amorphous Materials, Inc. (Houston, TX, USA), with an average diameter of 20 nm. ZnO NP suspensions were purchased from Alfa Aesar (Ward Hill, MA, USA) with an average particle size of 70 ± 15 nm. The concentration of the original suspension was 12 M. Corn starch was purchased from a local grocery store. *E. coli* O157:H7 strains G5310, C7927, and 3055-93 were provided by the Food Microbiology Laboratory at the University of Missouri, Columbia, MO. Tryptic soy broth (TSB) used to activate stock cultures as well as incubate fresh cultures were purchased from Difco Labs (BD Diagnostic Systems, Sparks, MD, USA). Fresh *E. coli* O157:H7 cultures were prepared by transferring 100 µL of each culture into separate tubes of 10 mL TSB and incubating for 18 to 20 h at 37°C (~ 10^{9} CFU/mL). Each strain was then mixed together, harvested by centrifugation (5000 g) at 4°C, washed and resuspended in 1% peptone water to yield a final level of ~ 10^{8} CFU/mL.

3.2 Effect of ZnO NPs on the growth of *E. coli* O157:H7 in TSB

E. coli O157:H7 cultures incubated overnight in TSB broth were inoculated into four flasks of TSB broths with a final concentration of approximately 10^{7} CFU/mL. Three of those four flasks contained 100 mL TSB broth with concentrations of ZnO NP suspensions at 0, 3, 6, and 12 mM, while the fourth flask contained 99 mL TSB broth and

1 mL ZnO NP-free solution. The NP-free solution was prepared by filtering ZnO NP suspension using a syringe assembled with anodisc inorganic membranes (20 nm pore size, Whatman Inc., Clifton, NJ, USA). Both the ZnO NP suspensions and the NP-free solutions were added to TSB before autoclaving.

Inoculated TSB containing ZnO NP suspensions and NP-free solution were incubated at 37 ^{o}C in a shaking incubator (G24, New Brunswick Scientific Inc., Edison, NJ, USA). The reason of using shaking incubator was to avoid the aggregation of ZnO NPs in the broth and to allow a consistent contact between *E. coli* O157:H7 cells and ZnO NPs. The growth of *E. coli* O157:H7 was monitored by checking the optical density value at 600 nm (OD_{600}) (UV-1650 PC, Suzhou Instruments Manufacturing Co. Ltd, Suzhou, China) and plating 1 mL samples on plate count agar (PCA) (Difco Labs, BD Diagnostic Systems, Sparks, MD, USA) at 0, 1, 2, 3, 4, 6, 8, 24, 48, 72, and 96 h. Uninoculated TSB containing 0, 6, and 12 mM of ZnO NP suspensions and ZnO NP-free solution were used as the control baseline for their corresponding inoculated broths during the OD value checking.

3.3 Preparation of yam starch films

A formula of 1.5 mL glycerol and 5 g yam starch (Mayushan Foods Co., LTD) mixed in 100 mL distilled water was selected for obtaining yam starch films. The amounts of starch and glycerol used were determined after preliminary tests (data not shown). ZnO NP suspensions (12 M) were added to the film-forming solution to make ZnO NPs concentrations of 0, 6 and 12 mM. The mixtures were heated to boiling temperature and maintained for 2 min with continuous stirring and then sterilized by autoclaving.

Films were prepared by casting approximately 25 mL of the cooled gelatinized solution to a polystyrene petri dish (Fisher Scientific, IL, USA) in a Biosafety Level 2 fume hood, followed by air-drying for 20 h. The resulted translucent films which could be easily peeled off from petri dishes were about 0.2 mm in thickness.

3.4 Effect of yam starch film with ZnO NPs on the growth of *E. coli* O157:H7 in beef cuts

Top sirloin beef steaks were purchased from a local supermarket. Corers were used to cut steaks into small cylinder-shaped pieces. Beef cuts were then dipped in fresh *E. coli* O157:H7 culture suspensions prepared as mentioned above to a depth of 0.6 cm for 2 min. A 15 min drip-drying step was followed and beef cuts were subsequently wrapped with yam starch films containing 0, 6 or 12 mM ZnO NPs. Beef cuts without *E. coli* O157:H7 inoculation and inoculated beef cuts without wrapping with yam starch films were selected as controls. All samples were kept at 4°C. Plate counting was performed at time intervals of 0, 2, 4, 6 and 8 days to monitor the growth of *E. coli* O157:H7 under different treatments. Each beef cut was weighed before placing in a stomacher bag with 1% peptone water to reach a 1:9 ratio (w/w). After 2 min of stomaching, 1 mL of the suspension was subject to plate-counting. Three replicate experiments were performed while treatments were duplicated within each replication.

3.5 Size of ZnO NPs powder

The size of ZnO NP powders were measured following the manufacturer's instructions. A 0.01% (m/v) suspension was prepared by adding 0.01 g ZnO NP powders

to 100 mL 95% ethanol and sonicating for 20 min. The resulted ZnO NP suspension was subject to standard TEM tests. Size distribution of ZnO NPs was obtained by analyzing TEM images using ImageJ software available at http://rsb.info.nih.gov/ij/.

3.6 Detection and characterization of ZnO NPs in corn starch

ZnO NPs were added to 7.5 g corn starch at concentrations of 0.1 and 0.5% (w/w). Samples were mixed thoroughly and transferred to crucibles, followed by incinerating in a furnace at 750°C for 16 h. The temperature and time of incineration were specifically designed high and long enough to burn off organic components, but low and short enough not to influence the inorganic components since the melting point of ZnO is 1975°C. Ashes were subsequently collected. Standard SEM and EDS were used to locate the nanoparticles and identify the elemental composition of the specimen. Prior to analyzing, samples were mounted onto SEM stubs and coated with a thin layer of carbon to enhance the conductivity of the specimen.

The feasibility of detecting and quantifying low concentrations of ZnO NPs in food samples was studied by inductively coupled plasma atomic emission spectroscopy (ICP-OES). ZnO NPs were artificially added to corn starch at concentrations of 0, 0.05, 0.1, 0.25, 0.5, 0.75, and 1% w/w (Table 3.1). Samples were evenly mixed before submitted to ICP-OES analysis (Chapman 1961) where 0.5 g of each sample was placed in a 10-mL crucible which was then transferred to a cool muffle furnace. Samples were then muffled at 500°C for ~2.5 h. After ashes were cooled to room temperature, 10 mL of 6 N HCl was added into each crucible to dissolve the ashes. The resulted suspension was

transferred to a glass tube and diluted to the 50 mL mark with DI water and mixed thoroughly. A Whatman No. 42 filter paper was used to filter the suspension. The filtered solution was analyzed for element determination (P, K, Ca, Mg, Na, Cu, Ag, Zn, and Ti) by ICP-OES. The quantity of ZnO NPs in the original sample was calculated based on the amount of Zn element detected. All experiments were replicated twice.

Table 3.1 Formulations of ICP-OES samples

ZnO NPs percentage (%)	ZnO NPs powder (g)	Corn starch (g)
0	0	5
0.05	0.0025	5
0.1	0.005	5
0.25	0.0125	5
0.5	0.025	5
0.75	0.0375	5
1	0.05	5

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Effect of ZnO NPs on the growth of *E. coli* O157:H7 in TSB

Our preliminary studies showed that ZnO NPs at 3 mM showed no significant inhibitory effect on the growth of *E. coli* O157:H7 in TSB. Figure 4.1 shows the OD_{600} value curves of *E. coli* O157:H7 in four flasks. The number of *E. coli* O157:H7 cells grew as the incubation time increased, as indicated by the increasing OD_{600} values. However, there were clear differences in the growth curves between four treatments. To begin with, the growth of *E. coli* O157:H7 in TSB with ZnO NP-free solution and TSB without ZnO NPs exhibited very similar growth patterns, which were different from the growth curves of the other two groups (TSB with 6 mM ZnO NPs and TSB with 12 mM ZnO NPs). As the concentrations of ZnO NPs in TSB broths increased to 6 and 12 mmol/L, the growth of *E. coli* O157:H7 was dramatically inhibited. One possible explanation is that ZnO NPs have antimicrobial functions and inhibited the growth of *E. coli* O157:H7 cells. Another possible reason is that ZnO NPs suspension was white in color, which affected the reading of the OD values. To further study the mechanisms, microbial plate count method was used to investigate the growth of *E. coli* O157:H7 cells.

Figure 4.2 shows the growth curves of *E. coli* O157:H7 by plating 1 mL of samples from each flask at time intervals of 0, 1, 2, 3, 4, 6, 8, 24, 48, 72, 96 h. A drastic decrease in the plate count numbers was observed during the first 3-h incubation in treatments

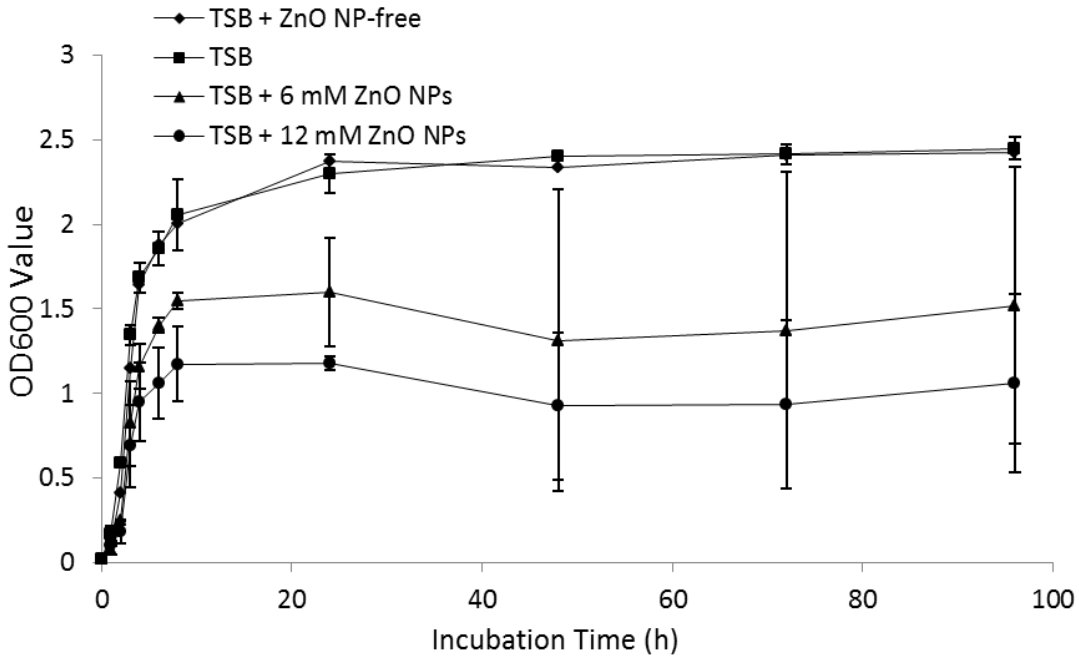

Figure 4.1 OD_{600} value curves of *E. coli* O157:H7 in four treatments

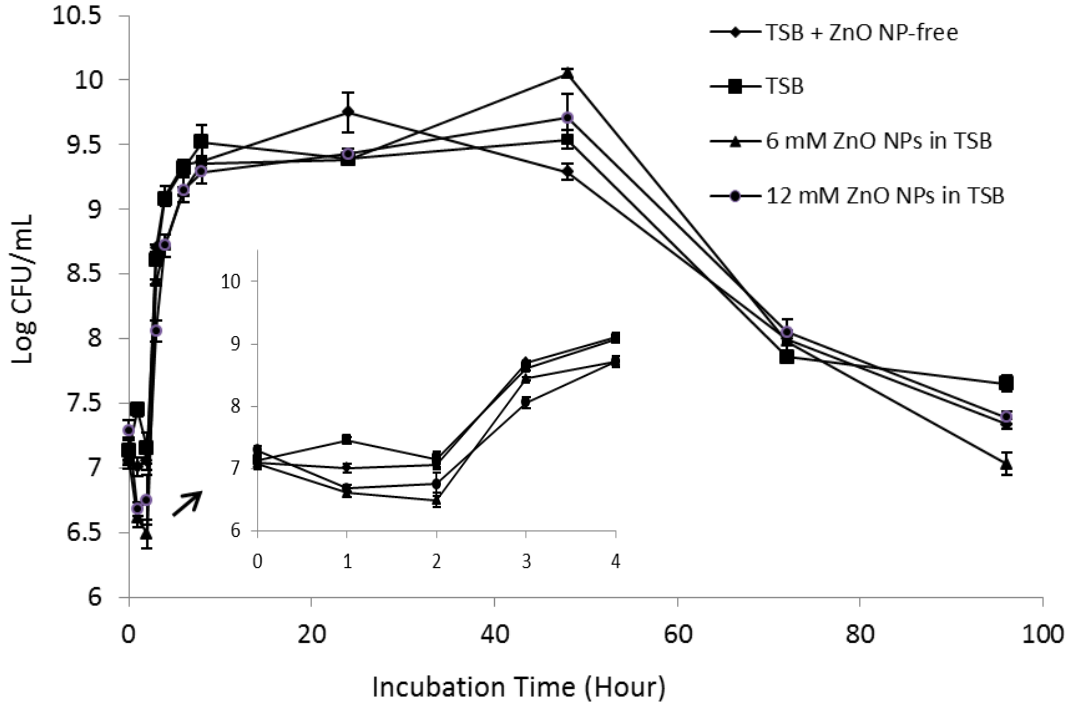

Figure 4.2 Effect of ZnO NPs on the growth of *E. coli* O157:H7 in TSB

with 6 and 12 mM ZnO NPs. After 3 h, the cell numbers in these two groups picked up slowly. The plate count numbers from all treatments were at the same level after 12-h incubation or longer. This suggests that besides particle size and concentration as previous studies indicated, time also affected the antibacterial activity of ZnO NPs, which should be taken into consideration in further studies. In addition, by comparing the OD_{600} value and the plate count numbers, it is worth noticing that the approach of examining OD_{600} value did not accurately monitor the actual growth curve of *E. coli* O157:H7 as the approach of plate counting method. It was possible that the strong absorbance of ZnO NPs at 600 nm wavelength interfered the reading of OD_{600} value and therefore resulted in less trustworthy growth patterns.

4.2 Effect of yam starch films with ZnO NPs on the growth of *E. coli* O157:H7 in beef

Figure 4.3 demonstrates the growth curves of *E. coli* O157:H7 in beef cuts wrapped with yam starch films containing different concentrations of ZnO NPs. Samples were kept at 4°C to simulate the conditions under which beef products are generally stored before consumption. Overall, plasticized yam starch films with 12 mM and 6 mM showed a 0.5-log reduction of the growth of *E. coli* O157:H7 over the controls. The results presented in Figure 4.3 are the average of three replications of which the growth of *E. coli* O157:H7 were all inhibited to some extent, with the results of replications 1 and 3 being the most obvious (~ 1 log reduction for replication 1 and 3). Within each replication, films with 12 mM ZnO NPs resulted in the most growth reduction of all four treatments; films with 6 mM ZnO NPs resulted in the second most. This was in agreement with the results of some previous studies (Brayner and others 2006; Jin and others 2009), which showed that the higher the concentration of ZnO NPs, the greater the growth reduction. However, the effects of yam starch films with 6 or 12 mM ZnO NPs were not significantly different from that of treatment without films or films without ZnO NPs ($p > 0.05$). This was most likely due to the low storage temperature (4°C), which considerably lowered the growth rate of *E. coli* O157:H7, a mesophilic bacterium, in the first place. Consequently, antibacterial activity of ZnO NPs appeared less effective than at a more optimum growing temperature, such as at 37°C.

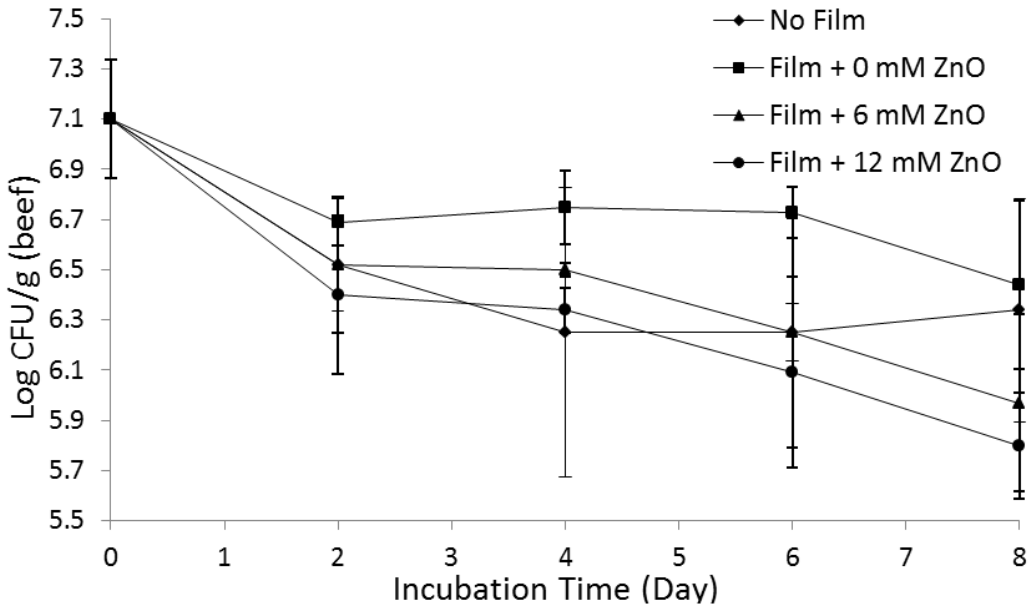

Figure 4.3 Antibacterial effects of yam starch films containing different concentrations of ZnO NPs on the growth of *E. coli* O157:H7 in beef

Storage time proved to have a significant impact on the growth curve of *E. coli* O157:H7 ($p < 0.05$), as the cell counts decreased gradually with incubation time. As seen in Figure 4.4, the antimicrobial effects of all four treatments were interpreted by survival rate. It is worth noticing that during the first two-day period, viable cells of *E. coli* O157:H7 dramatically dropped, recording a 60 to 80% decrease in survival rate. After that, cell counts of *E. coli* O157:H7 in all four groups continued to fall, but at a steadier and slower rate. This indicates that the antimicrobial activity of ZnO NPs became less effective over time, a theory that was supported by our study of the effect of ZnO NPs on the growth of *E. coli* O157:H7 in TSB.

Another set of experiments was carried out with the addition of vacuum packaging in the hope of creating a hurdle effect. It was expected the *E. coli* O157:H7 growth reduction effect would be enhanced to a higher level. However, no significant differences were observed compared with that of treatments without vacuum packaging (Figure 4.5). This phenomenon, nonetheless, could be explained by the fact that, according to previous studies, yam starch films are exceptional oxygen barriers because of their tightly packed, ordered hydrogen-bonded network structure and low solubility (Mali and others 2005), which partly accomplished the purpose of introducing vacuum packaging.

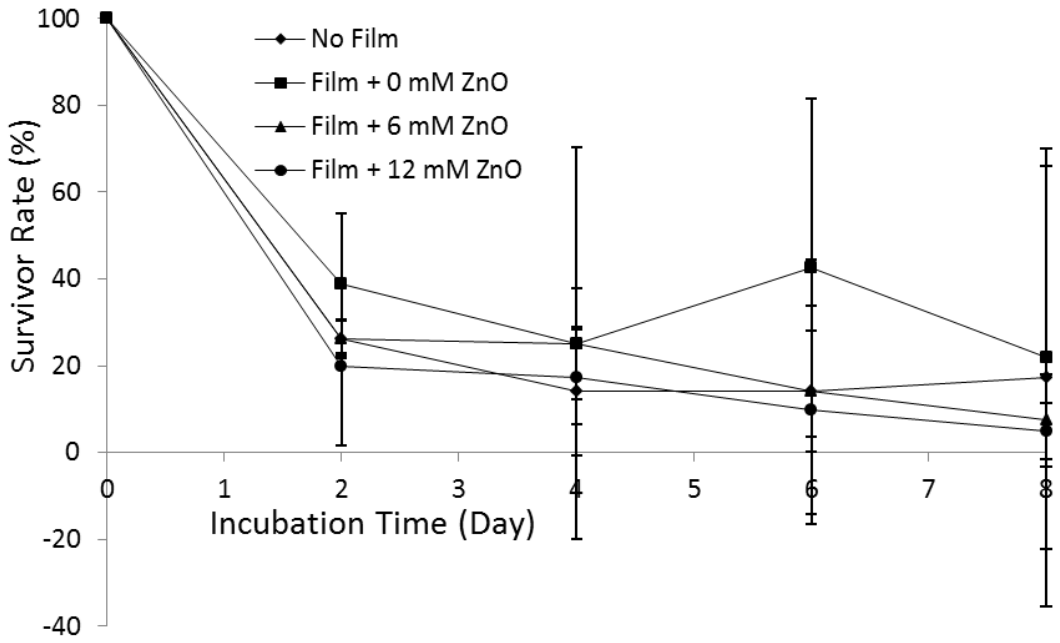

Figure 4.4 Survival rate of *E. coli* O157:H7 in beef wrapped with yam starch films containing various concentrations of ZnO NPs

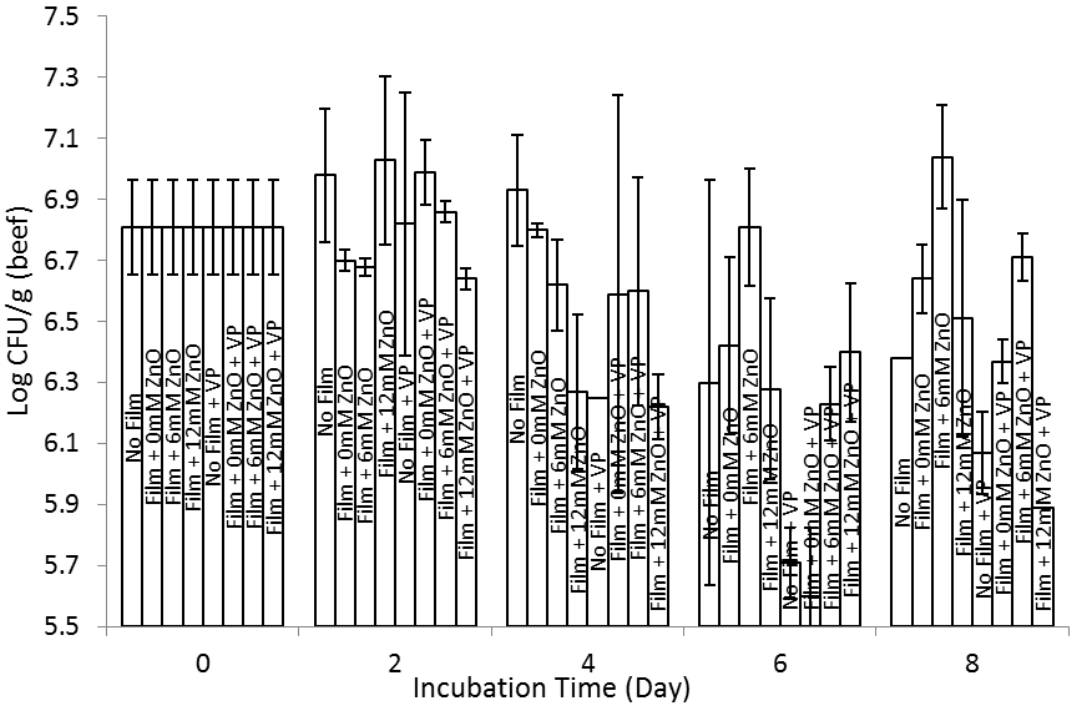

Figure 4.5 Antibacterial effects of yam starch films containing different concentrations of ZnO NPs on the growth of *E. coli* O157:H7 in beef with vacuum packaging

4.3 Characterization of ZnO NPs

The size and morphology of ZnO NPs was investigated by suspending 0.01 g of the ZnO NPs in 100 mL 95% ethanol and sonicating the suspension for 20 min. The amount of ZnO NPs added and the preparation procedures were instructed by the manufacturer. The ZnO NPs observed under TEM (Figure 4.6) were uniform in size. Agglomeration was common, due to the ultra-small size, high surface energy of ZnO NPs and electric double layer (EDL) compression (Zhang and others 2008). Most ZnO NPs were either round- or oval-shaped. Upon further analysis of the TEM image by ImageJ (Choi and Hu 2008), a histogram of the size distribution of ZnO NPs was generated (Figure 4.7). The majority of ZnO NPs measured fell in the range of 35 to 50 nm in diameter with an average size of 44.6 nm in diameter, which is in relatively close accordance with the advertised size of the product (APS: 20 nm). The results suggest that with the use of ImageJ, TEM is efficient and relatively accurate in determining the size of nanoparticles and could potentially be established as a standard procedure, although the concentration of nanoparticles and the type of organic solvent might differ as target matter varies. Further modification to the methodology is needed to improve the accuracy and accelerate the procedure.

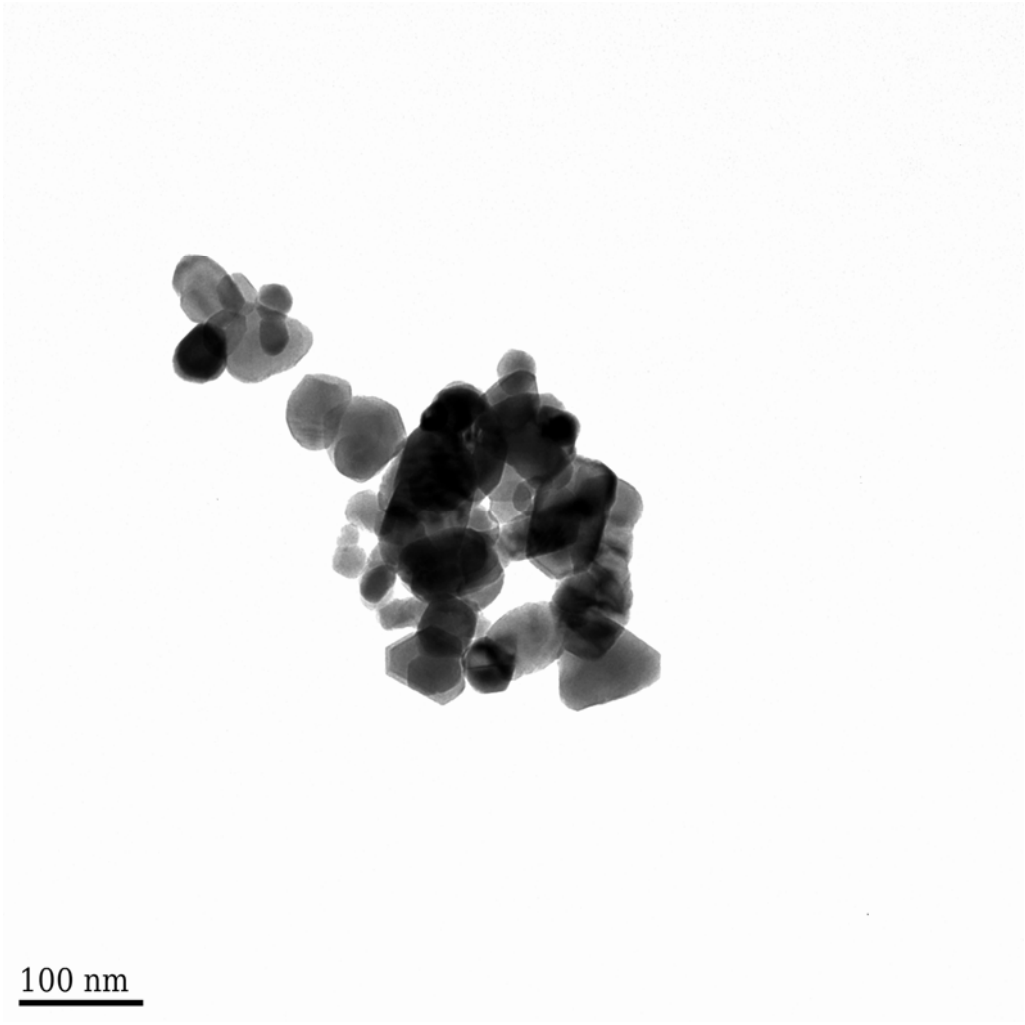

Figure 4.6 TEM image of ZnO nanoparticles suspended in 95% ethanol after a 20-minute sonication

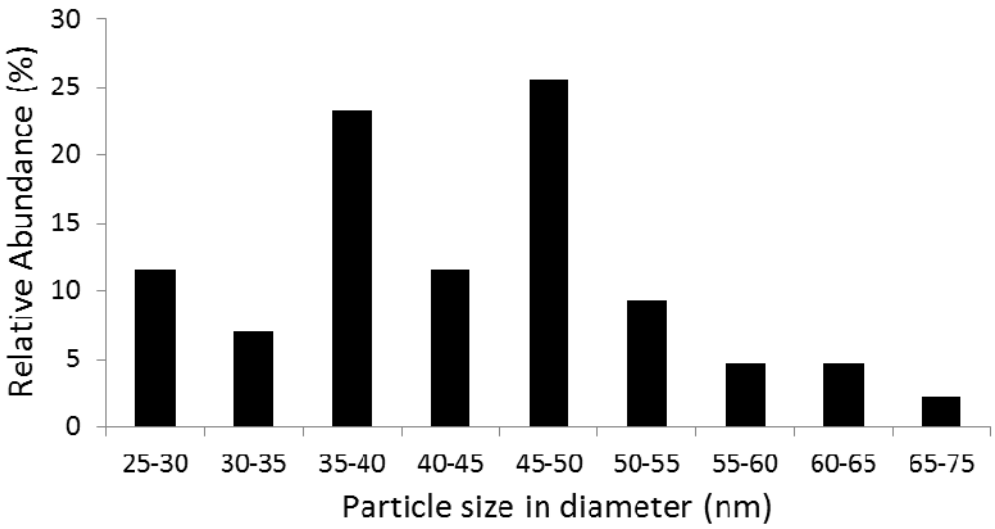

Figure 4.7 Size distributions of ZnO NPs with an average size of 44.6 nm in diameter

4.4 Identification of ZnO NPs in corn starch

ZnO NPs in corn starch at 0.1 and 0.5% w/w were mixed in crucibles and incinerated into ashes in a furnace. SEM micrographs were taken of the ashes. Upon elaborative comparison between the images of ashes with different ZnO NPs concentration, a 0.5% group with better contrast and particle distribution was chosen for future EDS analysis. The observed particles on the image can be generally classified into two groups regarding their sizes, with one ranging from 20 to 200 nm in diameter, and the other one over 200 nm in diameter. The former group was believed to consist of most ZnO NPs and the aggregation of them, which also caused the discrepancy to the advertised size at some spots. The latter group with the larger particle size was predicted to be contributed by corn starch. Because no artificial scattering procedure was involved, the occurrence of aggregation seen in SEM images was significantly higher than that seen in TEM images. The SEM image of the area with the majority presumably being ZnO NPs and their clusters is shown in Figure 4.8.

Energy dispersive spectroscopy (EDS) is a common technique for the analysis of elemental composition of a specimen. It is also capable of generating a map of multiple chemical elements of interest at a specifically assigned spot. Information such as relative or absolute concentration of all elements can be determined (Zheng and others 2011). Scanning electron microscopy (SEM) coupled with EDS has been successfully used to retrieve the relative distribution of complementary or correlating elements of tested

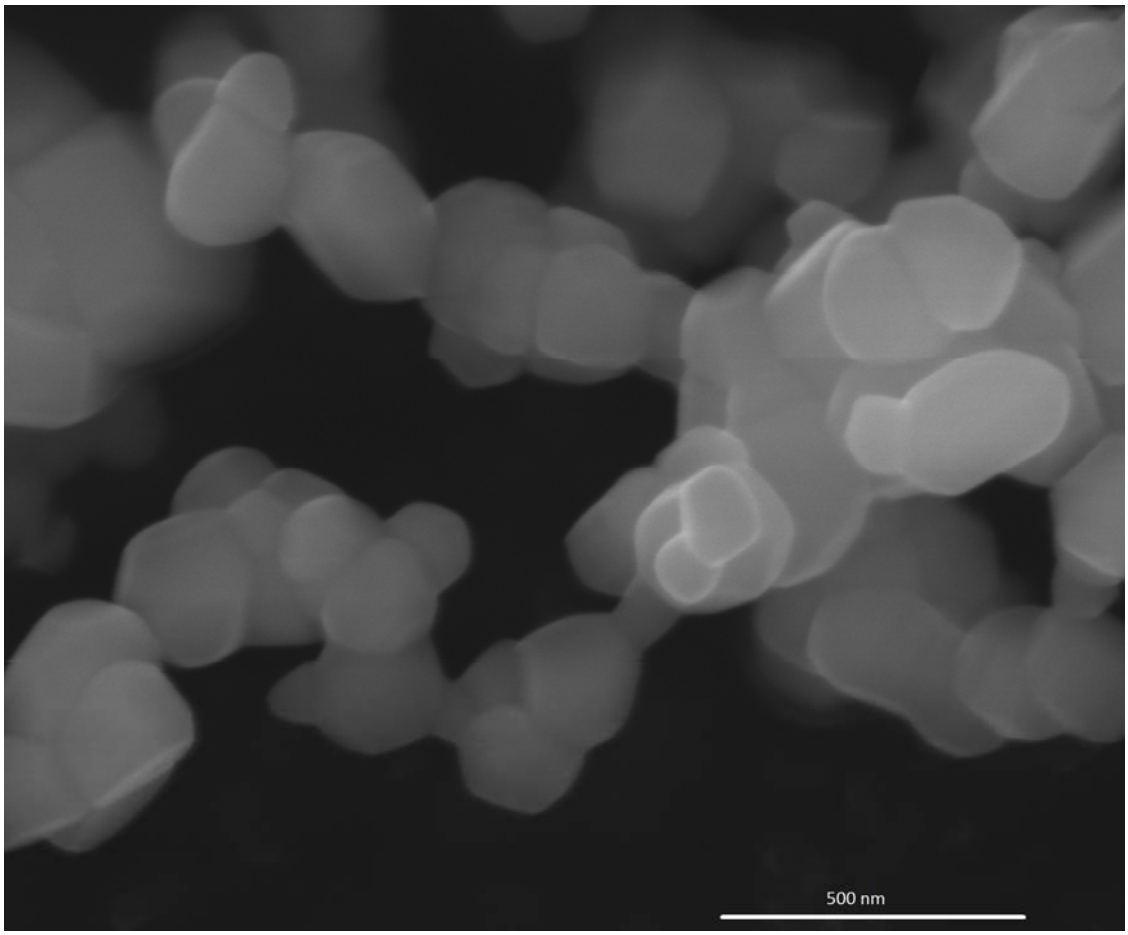

Figure 4.8 SEM image of incinerated corn starch ashes containing ZnO NPs

samples. It was effective in locating and identifying silver nanoparticles in nitrifying bacterial cells (Choi and others 2009). Figure 4.9 illustrates the SEM-EDS element analysis of ashes acquired from corn starch containing ZnO NPs. The visually bright spots on the SEM image contained more Zn element than dark spots according to EDS results (data not shown). Four locations where one implies corn starch and three other where the majority of particles belong to group one (40 – 200 nm in diameter) were selected and analyzed by selecting the point shooting mode. Results showed that at location 1, no zinc element was identified, suggesting corn starch was the dominating source. While at the other three spots, a relatively large count of zinc element was observed, indicating the presence of ZnO NPs. The relative atomic and weight percentage of each element at each detection point are shown in Tables 4.1 and 4.2. Zn was abundant at three of the four spots, as in accordance with results shown in Figure 4.9. Carbon was present at all four spots, indicating the presence of an overlap of ZnO NPs and particles from corn starch ashes. The atomic ratio of zinc and oxygen is close to 1 to 1 at three locations where zinc was identified. This suggested that ZnO NPs could be successfully identified despite that they were not homogeneously dispersed in corn starch ashes. The *K* and *L* in the Tables following each element represent different orbits of the corresponding atoms. The presence of element molybdenum (Mo) was unexpected, which was most likely contributed by the sample holder. Another possibility is that the characteristic peak of Mo was very similar to the characteristic peaks of other elements in the sample and the software gave the incorrect identification.

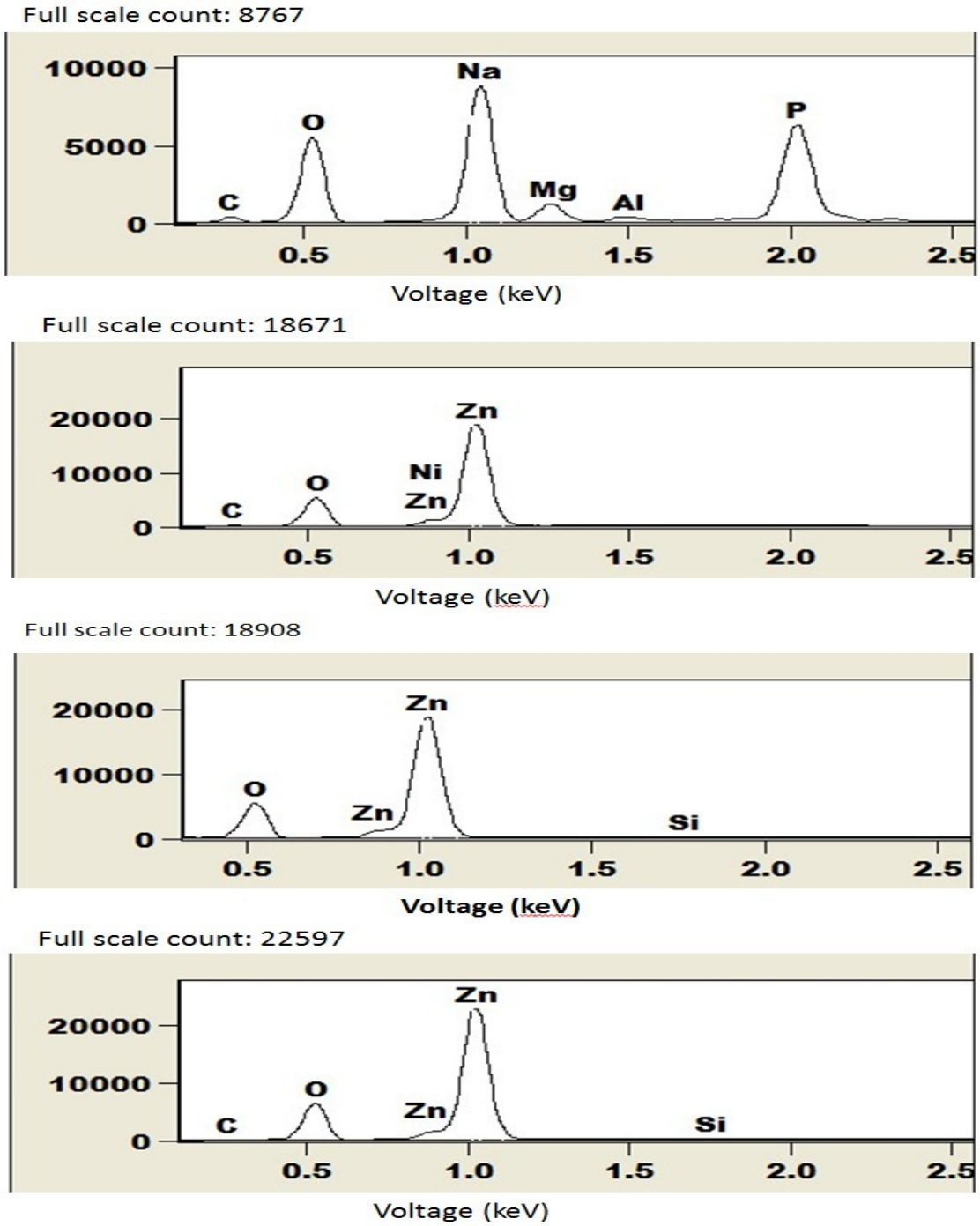

Figure 4.9 Energy-dispersive X-ray spectroscopy spectrum of ashes from corn starch containing ZnO NPs (0.5% w/w)

Table 4.1 Relative atomic percentage of identified elements at each spot

Location	C-K	O-K	Na-K	Mg-K	Al-K	Si-K	P-K	K-K	Ca-K	Ni-L	Zn-K	Mo-L
1	9.14	47.65	23.04	2.72	0.33	ND	15.44	0.21	1.26	ND	ND	0.2
2	9.56	52.31	ND	ND	ND	ND	ND	ND	ND	0	38.14	ND
3	24.27	45.01	ND	ND	ND	0.14	ND	ND	ND	ND	30.59	ND
4	3.93	54.97	ND	ND	ND	0.16	ND	ND	ND	ND	40.95	ND

*ND: not detected

Table 4.2 Relative weight percentage of identified elements at each spot

Location	C-K	O-K	Na-K	Mg-K	Al-K	Si-K	P-K	K-K	Ca-K	Ni-L	Zn-K	Mo-L
1	5.04	37.05	26.05	3.25	0.44	ND	23.52	0.4	2.49	ND	ND	0.96
2	3.33	24.29	ND	ND	ND	ND	ND	ND	ND	0	72.38	ND
3	9.67	23.89	ND	ND	ND	0.13	ND	ND	ND	ND	66.32	ND
4	1.31	24.37	ND	ND	ND	0.12	ND	ND	ND	ND	74.2	ND

*ND: not detected

4.5 Quantification of ZnO NPs in corn starch

Table 4.3 shows the results of ICP-OES analysis of corn starch containing different concentrations of ZnO NPs. The presence of extraneous Zn, contributed by added ZnO NPs, was detected at all selected levels. The detection limit in this study was determined to be 0.05% w/w. Since aggregation of ZnO NPs was inevitable, it was predictable that the detection limit could be improved, with more thorough mixing or means that can better pinpoint the location of ZnO NPs in corn starch.

Except for the Zn naturally contained in corn starch, the rest of Zn could only be contributed by added ZnO NPs. Consequently, the amount of Zn element detected was used to calculate the amount of ZnO NPs put in originally. A calibration curve was established to correlate between the actual values and the calculated values (Figure 4.10). Although the calculated values were not completely equivalent to the actual values, the quantification result was encouraging with the R^2 value of the curve being 0.984, indicating that the results were consistently accurate in reflecting the actual amount. Evidently, ICP-OES shows the promise to be used to quantify ENPs in foods and is feasible for potential applications in the food industry.

Table 4.3 ICP-OES results of corn starch contaminated with ZnO nanoparticles

Samples	Concentration of elements (ppm)							%[§]
	P	K	Ca	Mg	Na	Cu	Zn	
Pure CS	145	53	471.5	110	199	2.5	2.6	0
CS+0.05% ZnO	141	26	507	115	232	1.8	514.7	0.06
CS+0.1% ZnO	145.5	52	488.5	111.5	193.3	1.9	1376.5	0.17
CS+0.25% ZnO	147	74	362.5	101.5	207.5	2.3	2993	0.37
CS+0.5% ZnO	152	48	481.5	110	198.8	1.7	5970	0.74
CS+0.75% ZnO	146.5	74.5	383.5	103.5	157.3	1.9	10084.5	1.24
CS+1% ZnO	150	81	386	94	163.9	1.6	11140	1.39

*CS: corn starch; [§]: Calculated percent Zn originated from ZnO NPs.

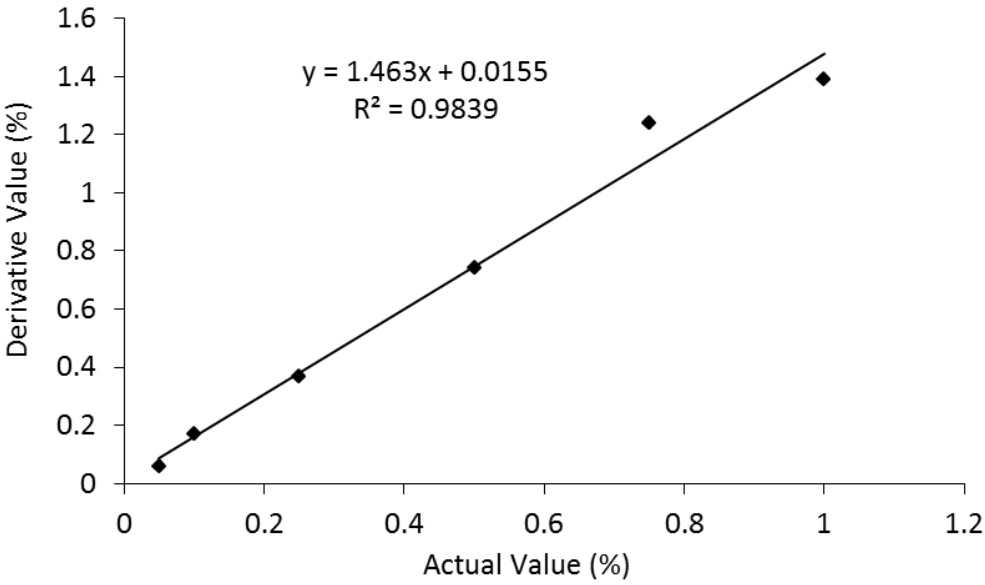

Figure 4.10 Calibration curve of the correlation between actual values (spiked amount) and derivative values (ICP-OES measurement) of ZnO NPs in corn starch

CHAPTER 5

CONCLUSIONS

5.1 Summary of the study

The rapid development of engineered nanoparticles (ENPs) has increased their applications in medical, engineering, cosmetic, and food industry. However, the adverse effect of ENPs on environment, essential microorganisms, and human health has been suggested. The consequence of ingesting ENPs could be fatal to the host. Ironically, with the commercialization of nanomaterial-containing products, no regulation has yet to be established regarding the use of ENPs in foods and no systematic studies have been conducted on detection and characterization of ENPs contamination, leaving consumers' safety in jeopardy. In this study, a systematic approach was proposed in which a trace amount of ZnO NPs powder was detected and quantified by a combination of methods including EDS and ICP-OES in corn starch. The sizes, morphology, aggregation, and other properties of ENPs were investigated by SEM and TEM. ZnO NPs were successfully detected and identified by SEM-EDS. Aggregation of ENPs was observed under both SEM and TEM scopes, which was understandable due to the high specific surface area of ENPs. This phenomenon was inevitably encountered in practically every study involving ENPs or other ultrafine particles. However, the level of aggregation was significantly lowered by sonicating ZnO NPs powder in solvents such as ethanol or deionized water, as seen in the TEM images. Sizes were analyzed by running ImageJ on the TEM image where scattered nanoparticles were observed. Results were

comparably close to the advertised size of the product. In addition, morphology of the nanoparticles was revealed by SEM and TEM images.

ICP-OES was employed to quantify ZnO NPs in corn starch where they were artificially added to. The lowest detection level of ZnO NPs was estimated to be 0.05% in corn starch with a R^2 value of 0.984. Moreover, results of UV-Vis spectra analysis were similar to previous studies of ZnO NPs. These results demonstrate that contamination of ENPs in foods could be detected and characterized by a combination of techniques including TEM, EDS, ICP-OES and UV-Vis spectroscopy, although modifications are required to refine the accuracy and precision of the methodology.

Innovative packaging materials are urgently needed as foods that are rich in nutrients and nourishment suffer from quality and economical loss due to microbial spoilage and contamination. The antimicrobial activity of ZnO NPs has been proven, while plasticized yam starch films have proven to extend the shelf life of some vegetables and fruits. In light of these results, the combination of ZnO NPs and yam starch films were studied for its antibacterial effect on *E. coli* O157:H7 in beef.

To simulate the post-production conditions under which beef products were stored, samples were kept at 4°C after treatment. After 8 days, yam starch films with 6 and 12 mM ZnO NPs were able to cause a 0.5-log reduction of the growth of *E. coli* O157:H7 compared with the controls. The reduction reached as high as 2-log in one replication. The addition of vacuum packaging showed no significant difference in inhibition efficacy, which was reasonable since yam starch films were excellent barriers for oxygen and

carbon dioxide. Nonetheless, the results suggest that plasticized yam starch films containing ZnO NPs were capable of controlling the growth of *E. coli* O157:H7 in beef.

5.2 Direction for future studies

To better characterize unknown ENPs, aggregations of ENPs shall be minimized. Sonication treatment for ENPs-containing solutions showed promising results, but was not sufficient and might introduce cross-contamination. A better approach is needed to solve this problem.

With regard to accurately report the amount of ENPs in foods, homogeneity of the sample is crucial. This was challenging considering the amount of ENPs in contaminated foods is generally at trace-level. ICP-OES was able to quantify ENPs in corn starch consistently, although the accuracy was not satisfying. We predict that with a sound mixing procedure, the results would be greatly improved.

To further facilitate the antibacterial activity of plasticized yam starch films containing ZnO NPs, higher concentrations of ZnO NPs might be needed, while remaining in a practical and safe range. Additional natural antimicrobial agents could be adopted as well in combination with the use of ZnO NPs.

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