



Faculty of Sciences

Department of Biological Sciences and Biotechnology

**Investigation of the Efficacy of a ZnO-Starch
Nanocomposite Packaging to Prolong the Shelf life of
Fragaria ananassa (Strawberries)**

by

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Dedication

To my late father Mr David Chitena, I hope I have made you proud.

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List of Abbreviations

NPs	Nanoparticles
ZnO	Zinc Oxide
NC	Nanocomposite
ZnO-starch NC paper	Zinc oxide starch nanocomposite paper
FWHM	Full Width at Half Maximum
UV	Ultraviolet
XRD	X-Ray Diffraction
eV	Electron Volt
M	Molar
nm	Nanometer
°C	Degrees Celsius
CNT	Carbon nanotubes
SWNT	Single walled carbon nanotubes
MWNT	Multi walled carbon nanotubes
g/L	Gram per Liter
mg/L	Milligram per Liter
JCPDS	Joint Committee on Powder Diffraction Standards
COD	Crystallography Open Database
meV	Mega electron volt
MIC	Minimum Inhibitory Concentration
MHA	Mueller Hinton Agar
SDA	Sabouraud Dextrose Agar
FCR	Folin-Ciocalteu Reagent
TiO₂	Titanium dioxide
TEM	Transmission Electron Microscope
SEM	Scanning electron microscope
ROS	Reactive oxygen species
GSH	Glutathione
FTIR	Fourier transform Infra-red
CFU/g	Colony forming units per Gram

Abstract

Nanomaterials have been used recently in food packaging as antibacterial coatings because of their desirable antibacterial and antifungal properties. The use of antimicrobial packaging is to deter the growth of microorganisms responsible for spoilage or diseases in packaged foods, therefore prolonging their shelf life. In this study, potato peel starch and ZnO-starch nanocomposite paper (ZnO-starch NC paper) were developed and evaluated for antibacterial activity against *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and antifungal activity against the *Botrytis cinerea* fungi. The ZnO nanoparticles (ZnO NPs) were synthesized using the hydrothermal method whilst the ZnO-starch NCs were synthesized by adding the potato starch to the ZnO NPs. The synthesized ZnO nanomaterials and potato starch were characterized using X-ray diffractometry (XRD) and UV-Vis spectrophotometry (UV-Vis). The analysis showed that the nanomaterials were purely synthesized without the presence of impurities. The ZnO-starch NC paper was made by coating Whatman filter paper (size 3) with the ZnO-starch NC. The antibacterial/antifungal activity of the ZnO-starch NC paper showed that the nanomaterials were effective against the Gram-positive bacteria *S. aureus* and *B. subtilis* but not against the Gram-negative *E. coli* and *P. aeruginosa*. The ZnO-starch NC paper was used to package inoculated strawberries in plastic containers for 10 days at 4°C and 27°C, respectively. Quality assays on the incubated strawberries showed that the percentage weight loss and bacterial counts of the fruits packaged with the ZnO-starch NC paper at 4°C were lower than those packaged without the ZnO-starch NC paper. The fruits incubated with the NPs were seen to be more acceptable in appearance and sensory value than their respective controls. In conclusion, the ZnO-starch NC paper was able to prolong the shelf life of strawberries for longer at lower temperatures. Hence, this nanomaterial could be used to explore nano-packaging for prolonging the shelf life of fruits.

Chapter 1: Introduction

1.1 Introduction

There is a growing interest in nanotechnology and the ability to use nanoparticles (NPs) in various industries. NPs are defined as small particulates with a diameter less than 100 nm (Laurent *et al.*, 2010; Christian *et al.*, 2008). This gives them certain chemical and physical properties, which are significantly different from bulk materials e.g. mechanical strengths, lower melting points and higher specific surface areas (Khan *et al.*, 2019). Nanoparticles have a very small size hence they have a very large surface area, and they are highly mobile in their free state (Gatoo *et al.*, 2014; Jarvie, 2019). These properties make them desirable in commercial applications such as drug delivery, electronic device development and nano-packaging (Stark *et al.*, 2013; Shi *et al.*, 2013; Abbas *et al.*, 2021).

Nanocomposites (NCs) are polymers with small inorganic particles embedded in them, making them have a high aspect ratio (Sorrentino *et al.*, 2007). Numerous nanomaterials have been studied and are generally divided into zero-dimensional (carbon quantum dots, NPs, clusters), one-dimensional (nanowires, nanoribbons), two-dimensional (graphene, nanofilms, nanolayers), and three-dimensional (e.g., graphite, aerogels, diamond) nanomaterials (Sharma *et al.*, 2012). NCs exhibit special properties because of the NPs embedded in them. Furthermore, the NPs tend to agglomerate during the preparation stages and mixing them with an organic matter or polymer helps to enhance dispersal and prevent agglomeration (Sorrentino *et al.*, 2007; Lebogang, *et al.*, 2019).

There are various methods of synthesizing NPs such as the wet chemical (Guo *et al.*, 2018), precipitation (Ghorbani *et al.*, 2015), hydrothermal (Gan *et al.*, 2020) and sol-gel method (Parashar *et al.*, 2020). These methods produce different types of NPs with various sizes, shapes and orientations. The hydrothermal method is the most common and efficient method as it does not require any organic solvents. This method uses a mixture of chemicals that are heated in an autoclave for up to several days. It utilizes high or low temperatures ranging from 100 to 300°C as well as high or low pressure to produce NPs. Varying these parameters can lead to the production of NPs of different shapes and sizes (Hayashi and Yakuta., 2010; Abbas *et al.*, 2021; Gan *et al.*, 2020).

1.2 Literature review

1.2.1 Spoilage of strawberries

Strawberries (*Fragaria x ananassa*) are a perennial fruit that is very juicy and sweet but also tends to spoil very easily (Feliziani and Romanazzi, 2016). This is due to the intrinsic and extrinsic factors expressed by the fruit. Depending on these factors, fresh foods usually spoil faster and the most common way to keep fresh fruit fresh for longer is to use low temperature or chemical preservatives, which are not very effective. The common intrinsic factors of fresh strawberries include pH, water activity, redox potential as well as nutrient content (Moral *et al.*, 2017). Fresh strawberries have a low pH of about 3.2 – 4.5, which is usually too high for certain bacteria to proliferate. Some microbes such as acidophiles and certain species of fungi can adapt to the acidic environment and cause spoilage (Giampieri *et al.*, 2012). Moisture content or water activity (*aw*) is the amount of water that is free to be part of chemical reactions and assist in microbial growth. Strawberries usually have the optimal *aw* for microbial growth between 0.90 – 0.99. The *aw* can be reduced by adding sugar, salt or by drying the fruits (Barth *et al.*, 2010). Redox potential is the ability of the strawberry to accept (oxidizing) or donate (reducing) electrons. Redox potential is measured in millivolts and depends on the ripeness of the fruits. Strawberries have a redox potential of between 200 – 240 mV but as the fruits continue to respire the redox potential will decrease leading to the spoilage of the fruits (Moral *et al.*, 2017; Dilbaghi and Sharma, 2007). Strawberries contain certain nutrients that microbes can utilize for growth and spoilage. Nutrients such as vitamins, carbohydrates, amino acids and water can be used up by microbes for their growth. Different microbes need specific nutrients to grow, and some cannot grow in the presence of certain molecules such as polyphenols, which can be toxic to them. The nutrient content of the strawberries affects the rate of spoilage and the type of spoilage microbes that infect them (Giampieri *et al.*, 2012; Barth *et al.*, 2010).

The common extrinsic factors involved in the spoilage of fresh strawberries include temperature, use of pesticides or fungicides and humidity among others (Moral *et al.*, 2017). Strawberries must be kept at low temperatures or refrigerated to slow down the proliferation of microbes. Higher temperature causes the growth of microbes and spoilage to occur at a faster rate (Ragaert *et al.*, 2004). Gases such as O₂ and CO₂ affect the intrinsic factors such as redox potential hence they have a direct effect on spoilage. The levels of these gases can be manipulated to prevent the growth of some spoilage microbes which are sensitive to these gases e.g., CO₂ is toxic to obligate aerobes and can prevent their growth

(Barth *et al.*, 2010; Doyle and Sperber, 2009). Pesticides and fungicides are applied to strawberries to prevent the growth of spoilage microbes and prevent insects from eating the fruits causing lesions and intrusions thus introducing pathogens into the fruit, causing spoilage (Feliziani and Romanazzi, 2016; Petrasch *et al.*, 2019). Humidity is directly linked to temperature and *aw*. At low temperatures, high humidity occurs resulting in water vapor condensing in the fruit causing an increase in *aw* whilst during elevated temperatures, low humidity occurs leading to the formation of water vapor resulting in a lower *aw* in the fruits (Moral *et al.*, 2017). As a result of these factors, microbes such as *Shigella spp*, *Listeria monocytogenes* and *E. coli* 0157:H7 (Mahovic *et al.*, 2005; Siddiqi *et al.*, 2018; Liu *et al.*, 2009) can grow on strawberries. Certain fungi have also been isolated on strawberries such as *Botrytis spp*, *Torulopsis spp* and *Saccharomyces spp* (Ragaert *et al.*, 2004; Dennis and Davis, 2008).

Strawberries are generally more perishable especially after harvesting due to the growth of fungi such as *B. cinerea*, which causes the growth of grey mold (Figure 1.0A) (Feliziani and Romanazzi, 2016; Petrasch *et al.*, 2019). *B. cinerea* is part of the world's most common pathogen that infects a wide range of crops pre-harvest and post-harvest. After infection, the pathogens spread to nearby healthy fruit as well as other fresh foods causing major losses. Infections occur pre-harvest and can stay dormant until after harvesting as the fruit will be stored in places of higher humidity and low temperatures (0 – 4°C) (Zhang *et al.*, 2018). Fungi such as *Rhizopus* and *Mucor spp* (Figure 1.0B and C), cause diseases such as soft rot (Hauke *et al.*, 2004). This disease is hard to manage, and preventive measures are needed from planting to harvesting. Anthracnose is a disease caused by the genus *Colletotrichum* and leads to brown spots on the fruit (Figure 1.0D) (Feliziani and Romanazzi, 2016). The fungi produce spores that are difficult to remove from the fruit and they activate in favorable conditions post-harvest causing spoilage. These fungi take advantage of mechanical damage on the fruits such as lesions and bruises (Ragaert *et al.*, 2004; Feliziani and Romanazzi, 2016).

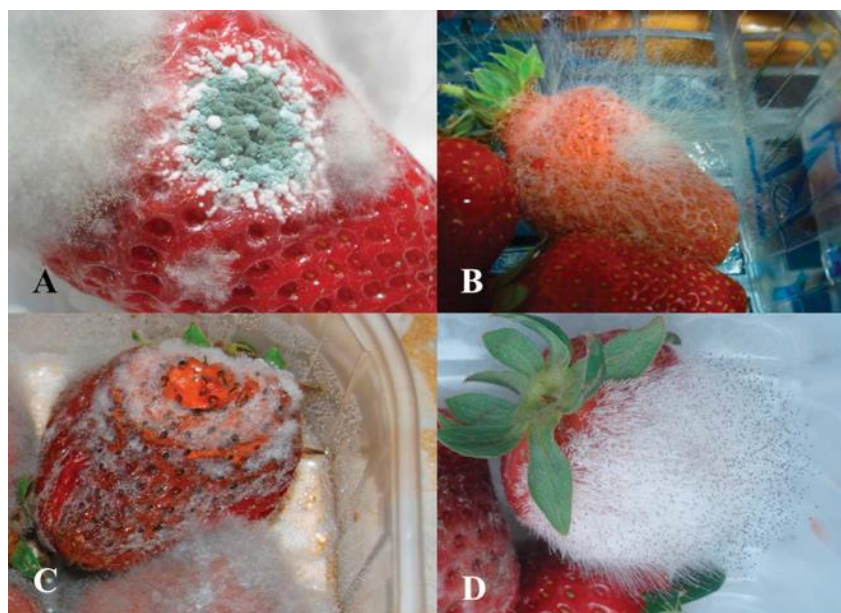


Figure 1.0. Strawberry spoilage by fungi. A) *Penicillium spp* (green) and *B. cinerea* (grey), B) *Rhizopus spp*, C) *Mucor spp*, D) *Colletotrichum spp* (extracted from Feliziani and Romanazzi, 2016).

Fungicides and pesticides are the most common methods of controlling pathogens but some of them have adverse side effects and are toxic to both humans and the environment, therefore it is important to find an alternative that is safer and environmentally friendly (Feliziani and Romanazzi, 2016). NPs such as ZnO can inhibit pathogens at very low concentrations and affect the soil less than regular pesticides. They offer a safer, cheap and natural alternative to current synthetic chemicals or antibiotics and they are highly effective as antibacterial, antifungal, antiparasitic and antiviral agents (Irkin and Esmer, 2015).

1.2.2 Use of NPs in industries and food packaging

NPs and nanomaterials have been used recently in many industries to develop new and more efficient technologies because of their desirable properties (Santos *et al.*, 2020; Simonsen *et al.*, 2018). There are different types of NPs of various sizes and characteristics which are available for industrial use. They are classified into organic, inorganic, and carbon-based NPs according to composition and how they are synthesized (Figure 1.1) (María–Mauricio *et al.*, 2018; Saleh, 2020). NPs have been used in the medical industry for drug delivery, diagnosis, treatment and the development of new medical tools (Abbas *et al.*, 2021; Berton *et al.*, 2001; Murthy, 2007). In the energy industry, they are used to develop new energy sources which are safer and environmentally friendly whilst in the electronics

industry they are used to advance computing technology leading to faster and smaller devices that have a larger storage capacity (Makwana *et al.*, 2015; Stark *et al.*, 2013). In the food industry, NPs have been used to develop nano-packaging which can be used to deter the growth of microorganisms responsible for spoilage or diseases in packaged foods (Weiss *et al.*, 2006; Shi *et al.*, 2013; Dobrucka and Ankiel, 2019). This is done by using biopolymers and NPs such as copper (Muthalakshmi *et al.*, 2017), magnesium oxide (Dela Rosa-Garcia *et al.*, 2018), silver (Ortega *et al.*, 2017) and zinc oxide (ZnO) (Abbas *et al.*, 2021), which because of their small size, different biological molecules can be immobilized on them leading to greater efficiency in antibacterial activity (Makwana *et al.*, 2015; Abbas *et al.*, 2021).

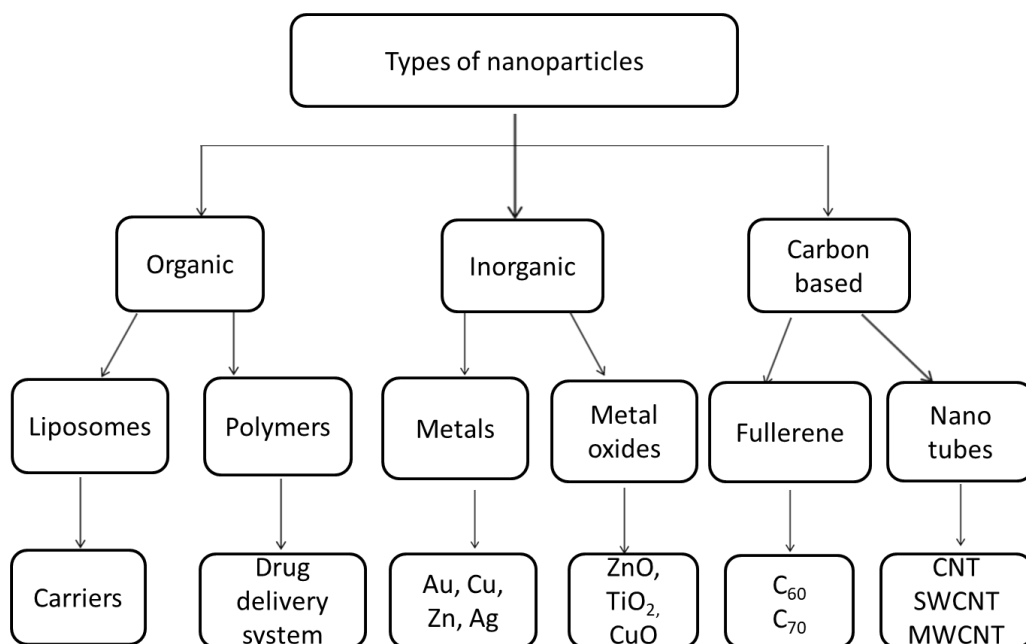


Figure 1.1. Classifications of NPs according to composition.¹

Over the years nano-packaging has evolved as they now can prolong the shelf life of food by excluding certain parameters such as moisture, light and gases, which can accelerate the spoilage process (Kuswandi, 2017; Bajpai *et al.*, 2018; Tavakoli *et al.*, 2017). There are different types of nano-packaging that fall under two categories, which are active and improved packaging. Active packaging can directly interact or be in contact with food therefore, providing a barrier between the food and external factors (Costa *et al.*, 2016;

¹ (CNT = Carbon nanotubes, SWNT = single walled carbon nanotubes and MWNT = multi walled carbon nanotubes).

Akbar and Anal, 2014). Improved packaging is when the nanomaterials are incorporated into a polymer matrix (such as starch or chitosan) to improve the gas barrier properties, block UV-rays, add strength, heat resistance and firmness (Kuswandi, 2017; Adeyeye, 2019; Chaudhary and Kumar, 2020). Nanoparticles such as silver (Durán *et al.*, 2010; Khalaf *et al.*, 2013) work by improving the internal environment of the food by breaking down gases such as ethylene gas, which cause food spoilage whilst Titanium oxide (TiO₂) was found to have oxygen scavenging properties (Close *et al.*, 2015). This study focuses on improved packaging whereby ZnO NPs were embedded into a polymer matrix using starch.

1.2.3 ZnO NPs toxicity and safety

As NPs have been used in different industries their toxicity has been a concern to the public. ZnO is listed as generally recognized as safe by the Food and Drug Administration and has been used as a food additive and supplement (Prasad, 2008; Wang *et al.*, 2017; Siddiqi *et al.*, 2018). ZnO NPs are also an essential trace element needed by the human body (Siddiqi *et al.*, 2018). Some studies have been done to assess ZnO NPs toxicity towards human cell lines. ZnO has been shown to have higher toxicity to some human cells such as human lung cancer and umbilical vein endothelial cells at concentrations of above 240 µmol/L after 14 days of incubation, which led to cell death (Chen *et al.*, 2016; Suh *et al.*, 2013). In vivo studies by Cross *et al* and Filipe *et al*, researched the effects of sunscreen lotions containing ZnO on the human skin. (Cross *et al.*, 2007; Filipe *et al.*, 2009). Electron microscopy results showed that ZnO NPs were not present in the deeper layers of the skin and there has not been any evidence on the absorption of ZnO through the skin till today (Wang *et al.*, 2017). Sharma *et al* and Ko *et al*, investigated the effects of ingesting ZnO orally (Sharma *et al*, 2012; Ko *et al.*, 2015). Their results showed that ingesting ZnO at concentrations of above 500 mg/kg/day leads to the NPs accumulating in vital organs resulting in cell death (Sharma *et al*, 2012; Ko *et al.*, 2015). The results of these studies show that for the NPs to have an adverse effect on human cells or confer toxicity, they must be ingested orally at high concentrations for an extended time to cause health problems. In commercial industries, NPs are used in very low concentrations and are embedded in the polymer matrix to reduce leaching of NPs as well as direct contact with any food substances. This means that the toxicity of ZnO in humans due to their use in commercial applications is highly improbable as no such evidence has been found (Wang *et al.*, 2017; Chen *et al.*, 2016).

1.2.4 Characterization of nanomaterials

The morphological characteristics of NPs are very important, and several techniques are currently used to characterize NPs. The most common methods are microscopic techniques such as scanning electron microscopy (SEM) and transmission electron microscopy (TEM) (Hall *et al.*, 2007; Mourdikoudis *et al.*, 2018; Vega-Baudrit *et al.*, 2019). These methods can provide information about the size and shape of the NPs. Small particles which are less than 1 nm can be viewed and information on the surface texture can be generated. Other techniques such as the X-ray diffractometer (XRD) give information on the composition and nature of the bonding materials as well as information about bulk properties. This method confirms the presence of NPs and the crystallite size or shape can be estimated using the Scherrer formula (Modena *et al.*, 2019; Kumar and Dixit, 2017). The UV-Vis spectrophotometer and Fourier Transform infra-red spectroscopy (FTIR) measure how much light materials can absorb. They are used to measure the vibrational characteristics of NPs (Khan *et al.*, 2019; Modena *et al.*, 2019). FTIR uses infra-red light to scan and test samples and identify polymeric or organic materials, surface composition and ligand binding (Mourdikoudis *et al.*, 2018; Titus *et al.*, 2019). In this study, we used the XRD and UV-Vis techniques to characterize the synthesized nanomaterials.

1.2.5 Properties of Zinc oxide

ZnO is defined as an II – VI semiconductor, as it is defined into the second and sixth groups of the periodic table. Being part of these groups' means it has very desirable properties such as a wide bandgap of about 3.37 eV in the near UV spectrum and a binding energy of about 60 meV at room temperature (Wang *et al.*, 2018; Gou, 2017). The wide bandgap affects the optical absorptivity and electrical conductivity of ZnO. The optical absorption is in the near UV region therefore on top of high antibacterial activity, it can also be used as a sunscreen agent in the cosmetic industry (Mazitova *et al.*, 2019; Smijs and Pavel, 2011). Other structures such as the rock salt (Figure 1.2A) and the zinc blende (Figure 1.2B) are formed under special conditions of synthesis. During synthesis, ZnO usually crystallizes into a hexagonal wurtzite structure, which is the most thermally stable structure (Figure 1.2C) (Özgür *et al.*, 2018). Through variation of temperature or pH, different structures can be synthesized, broadening the applications of ZnO, as novel structures can subsequently be created (Chauhan *et al.*, 2018; Wang *et al.*, 2018).

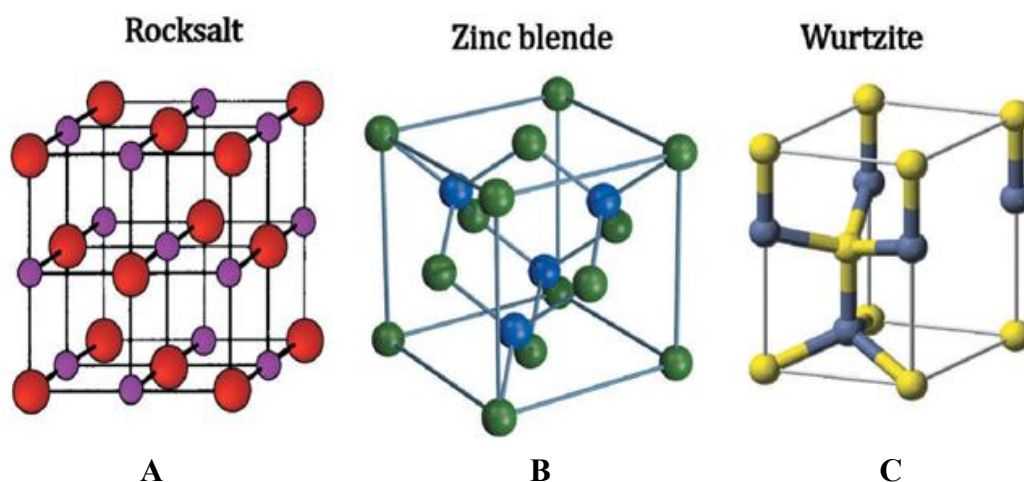


Figure 1.2. Different structures of ZnO A) rocksalt, B) zinc blende and C) wurtzite (modified from Özgür *et al.*, 2018).

ZnO is also desirable as it is active against both Gram-negative and Gram-positive bacteria and has been proved to be active against *Listeria spp*, *Salmonella spp*, *Shigella spp* as well as fungi such as *Penicillium spp* among others (Mahovic *et al.*, 2005; Ragaert *et al.*, 2004; Tayel *et al.*, 2011). Metal oxide NPs are more desirable as they are more stable and only small quantities are needed for effective antimicrobial activity (Siddiqi *et al.*, 2018; Jin *et al.*, 2009; Liu *et al.*, 2009). Furthermore, they also have been proven to be less toxic to humans than traditional NPs (Herbet *et al.*, 1996; Padmavathy and Vijayaraghavan, 2008).

1.2.6 Mode of action of NPs

NPs are known to attack several biomolecules in the pathogen cells therefore the bacterial strains are not able to develop resistant strains to the NPs unlike antibiotics which attack one biomolecule (Liu *et al.*, 2010; Shaikh *et al.*, 2019). Bacteria have been classified into two groups which are Gram-positive and Gram-negative. The Gram-negative bacteria have a cell structure consisting of an extra outer cell membrane and a thin single layered peptidoglycan whilst the Gram-positive cells have a thicker multilayered peptidoglycan layer without an extra cell membrane (Figure 1.3) (Slavin *et al.*, 2017; Neves *et al.*, 2019; Kuswandi, 2017). Some NPs are less sensitive to the Gram-positive bacteria due to the thick peptidoglycan layer which acts as a barrier against nanoparticle ions uptake (Ayala-Núñez *et al.*, 2008). In a study by Cavassin *et al*, multidrug-resistant bacteria such as vancomycin-resistant *Enterococcus spp*, oxacillin-resistant *S. aureus*, carbapenem-

resistant *P. aeruginosa*, copolymyxin B resistant *Acinetobacter baumannii* and carbapenem-resistant *Enterobacteriaceae* were exposed to citrate stabilized silver NPs (Cavassin *et al.*, 2015). The results showed that the antibacterial effect was stronger against the Gram-negative bacteria (carbapenem-resistant *P. aeruginosa*, copolymyxin B resistant *A. baumannii* and carbapenem-resistant *Enterobacteriaceae*) than the Gram-positive bacteria (vancomycin-resistant *Enterococcus spp* and oxacillin resistant *S. aureus*) (Cavassin *et al.*, 2015).

In another study by Dorobantu *et al.*, *P. aeruginosa* and *S. aureus* were exposed to mercaptoundecanoic acid and citrate capped silver NPs (Dorobantu *et al.*, 2015). After exposure, the results showed that the NPs were about 3 times more toxic towards the Gram-negative bacteria *P. aeruginosa* than the Gram-positive bacteria *S. aureus*, with both capped NPs failing to show any bactericidal activity after 4 hours of exposure (Dorobantu *et al.*, 2015). Some bacteria such as *Cupriavidus metallidurans CH34* have certain plasmids or genes which give the bacteria resistance to NPs hence most NPs do not affect them. Simon- Deckers *et al.*, investigated the effect of TiO₂ NPs on *E. coli* MG1655 and *C. metallidurans*. They found that the *E. coli* strain was sensitive to the TiO₂ NPs whilst the *C. metallidurans* strain was not as sensitive (Simon-Deckers *et al.*, 2009). The NPs were found to have accumulated in *E. coli* leading to death but in *C. metallidurans*, the NPs accumulated in the cells but did not have a fatal effect on the bacterial cells (Simon-Deckers *et al.*, 2009). This was found to be because of two plasmids present in *C. metallidurans* which are activated in the presence of NPs (Simon-Deckers *et al.*, 2009). The antifungal activity of ZnO NPs was investigated by He *et al.*, by exposing ZnO NPs to *B. cinerea* and *Penicillium expansum* (*P. expansum*) (He *et al.*, 2011). Different concentrations of NPs were used (0, 3, 6 and 12 mmol/l). The results showed that the NPs with concentrations of 3 mmol/l or more were most sensitive to *P. expansum* than *B. cinerea*. The ZnO NPs prevented the growth of conidiophores and spores in *P. expansum* whilst in *B. cinerea* they affected the cellular operations leading to deformation of the hypha surface (He *et al.*, 2011). The difference in reaction to the NPs was attributed to the morphological differences of the fungi as well as the concentration of the NPs used (He *et al.*, 2011).

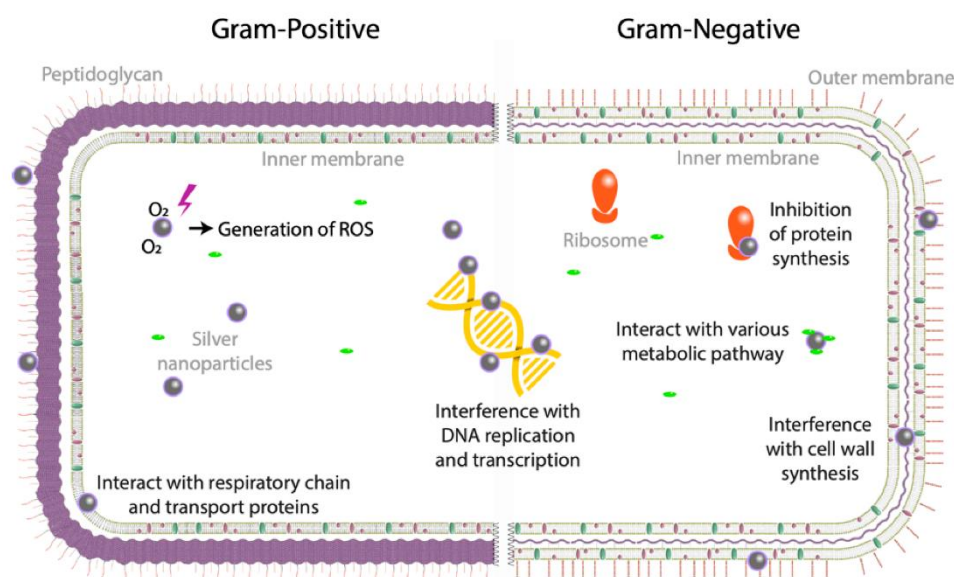


Figure 1.3. Diagrammatic presentation of the mode of action of ZnO NPs against Gram-positive and Gram-negative bacteria (extracted from Duval *et al.*, 2019).

The Gram-negative and Gram-positive bacteria both possess cell walls that are negatively charged and the most positively charged nanoparticle ions attach to and are taken up into the cell resulting in cellular damage and death (Slavin *et al.*, 2017; Neves *et al.*, 2019). Different NPs have different antibacterial activities as several studies have shown that ZnO NPs are more active against Gram-positive bacteria than Gram-negative bacteria. In a study by Emami-Karvani and Chehrizi, the effect of ZnO against the Gram-positive *S. aureus* and the Gram-negative *E. coli* was investigated using the minimum inhibitory concentration (MIC), time-dependent tests as well as the disc and well diffusion assays (Emami-Karvani and Chehrizi, 2012). Different concentrations of the ZnO nanoparticles were prepared (0.5, 1, 2, 4, 8 and 16 mg/ml) and tested against the bacteria. The results showed that the NPs were more bactericidal against the Gram-positive bacteria than the Gram-negative bacteria. The ZnO NPs at a higher concentration showed higher antibacterial activity than those of a lower concentration (Emami-Karvani and Chehrizi, 2012). Premanathan *et al.*, investigated the antibacterial activity of ZnO NPs against *E. coli*, *P. aeruginosa* and *S. aureus* using the MIC assay (Premanathan *et al.*, 2011). The results show that the Gram-negative bacteria (*E. coli* and *P. aeruginosa*) had a higher MIC level (500 µg/ml) than the Gram-positive *S. aureus* (125 µg/ml). This meant that the ZnO NPs are more effective against the Gram-positive bacteria at low concentrations but confer antibacterial activity against Gram-

negative bacteria at about 4 times the NPs concentration used against Gram-positive bacteria (Premanathan *et al.*, 2011).

The difference in antibacterial activity could be attributed to the mode of action of the ZnO NPs. NPs have been found to have different methods in which they kill or inhibit bacterial and fungal growth (Liu *et al.*, 2010; Shaikh *et al.*, 2019). For ZnO, the most common method is the production of ROS compounds such as H_2O_2 (Navale *et al.*, 2015; Xie *et al.*, 2011). This means that ZnO NPs are more effective against bacteria that are sensitive to ROS compounds but not to those resistant to reactive oxygen species (ROS) compounds (d'Água *et al.*, 2018). Gram-positive bacteria (*B. subtilis* and *S. aureus*) have been found to be more susceptible to ZnO than Gram-negative bacteria (*E. coli*) due to biofilm production and growth rate (Baek *et al.*, 2011; Hajipour *et al.*, 2012). Fast-growing bacteria are more susceptible to NPs than slow-growing bacteria and hence the effect of the NPs mostly depends on the type of strain involved (Hajipour *et al.*, 2012). The type of liquid in which the NPs are dispersed could affect the dissolution of the ZnO NPs. ZnO NPs form metal ions in solution which attach to bacterial cells and cause cell death but with the presence of certain compounds such as minerals, other ions or proteins, they can form complexes with them resulting in fewer ions being available for antibacterial activity (Bogdanovic *et al.*, 2014; Carrilo-Carrion *et al.*, 2014; Li *et al.*, 2011). This can affect the effectiveness of the NPs against certain resistant bacteria such as *E. coli* and *P. aeruginosa*.

1.2.6.1 Generation of reactive oxidative species (ROS)

ROS are produced in bacteria naturally through aerobic respiration whilst free radicals can also be produced by exposure to UV radiation. Species such as $OH\cdot$ (hydroxyl radicals), H_2O_2 (hydrogen peroxide) and O_2^- (superoxide anion radical) have been found to lead to oxidative stress causing damage to nucleotides and lipids in the bacterial cell (Figure 1.4). (Navale *et al.*, 2015; Xie *et al.*, 2011). NPs are very small in size, which gives them a larger surface area to volume ratio (Foster *et al.*, 2011; Manke *et al.*, 2013). This leads to the production of a larger number of ROS which leads to the damage of intracellular molecules such as proteins and organelles. The size of NPs also helps them to integrate into the cell system and participate in cellular reactions (Simon-Deckers *et al.*, 2009; Fu *et al.*, 2014). In a study by Navale *et al.*, ZnO NPs of different concentrations were synthesized and were exposed to *Salmonella typhimurium*, *S. aureus*, *Aspergillus flavus* and *Aspergillus*

fumigatus (Navale *et al.*, 2015). Investigations by UV photocatalysis and GSH oxidation (glutathione oxidation) methods showed that ZnO NPs produce ROS resulting in oxidative stress which leads to cell death (Navale *et al.*, 2015). Al Sharqi *et al.*, investigated the effects of silver NPs against *E. coli* and *S. aureus* where different concentrations of NPs were synthesized and exposed to the bacteria using visible blue light (Al Sharqi *et al.*, 2019). Using survival assay, the antibacterial efficacy of the NPs was determined, and ROS analysis was done on the bacteria which showed that the survival rate dropped to 14% for *E. coli* and 28% for *S. aureus*. This showed that the silver NPs are more toxic to *E. coli* than *S. aureus* and produce a high amount of ROS, which are responsible for its antimicrobial activity (Al Sharqi *et al.*, 2019).

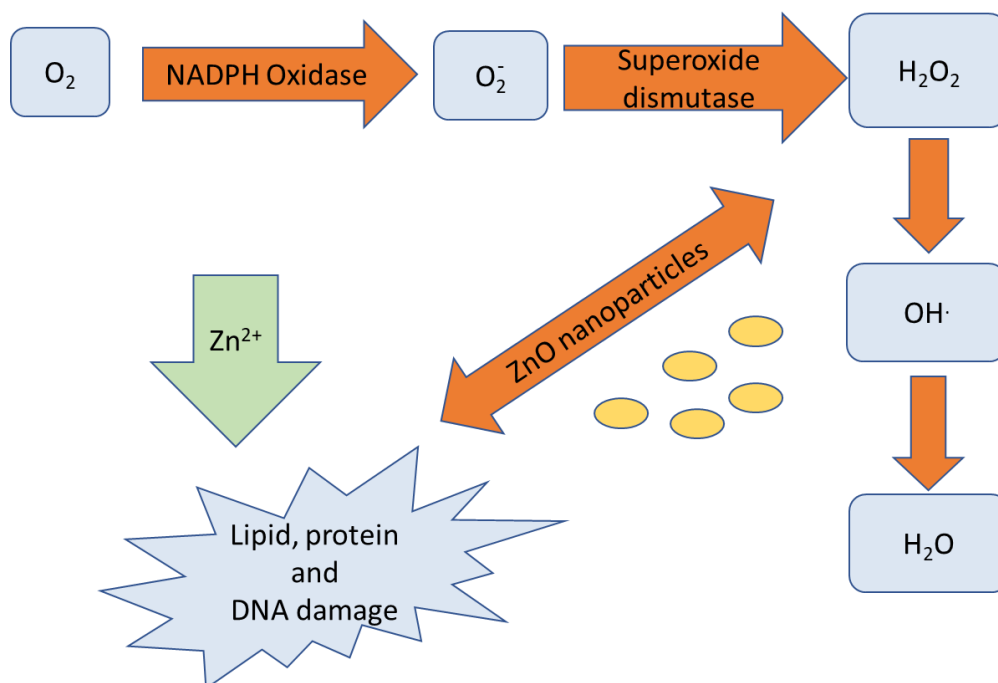


Figure 1.4. Role of ZnO NPs in the production of ROS.

1.2.6.2 Release of metal ions

Metal oxides release metal ions that are absorbed by cells, where they interact with internal organelles leading to cell death (Wang *et al.*, 2017). In a study by Wang *et al.*, ZnO and iron oxides (Fe_3O_4 and Fe_2O_3) NPs were dissolved in different test media and the dissolution value (EC_{50}) was determined. ZnO NPs were seen to have a very high dissolution value resulting in the Zn^{2+} ions being highly toxic against the test subject *Photobacterium phosphoreum* (Wang *et al.*, 2014). The Fe^{2+} ions were found to have a low dissolution value

below analytic quantification (Wang *et al.*, 2014). When ions such as Zn^{2+} (Mukha *et al.*, 2013) and Cu^{2+} (Navale *et al.*, 2015) build up in the cell they cause a change in the overall charge of the cell wall causing the cell membrane to become more porous and permeable. These metal ions move easily across the lipid bilayer and when taken into the cell can lead to ROS production, lipid peroxidation and protein oxidation leading to cell death (Bogdanovic *et al.*, 2014; Carrilo-Carrion *et al.*, 2014).

1.2.7 Properties of starch and its use in food packaging

Starch is a polysaccharide that has been used as an edible coating in meat products, fruits, vegetables and in some baking products as a protective barrier against lipids (Pagella *et al.*, 2002; Santos *et al.*, 2020). This material is easily available and is a cheap renewable material that can improve other materials when blended with them (Sorrentino *et al.*, 2007; Wesley *et al.*, 2014). On its own, starch is brittle and has poor mechanical performance hence it must first be chemically modified to make it stronger by blending it with other materials. When starch is utilized in making films or nanocomposites, its properties are dependent on the source of the starch as well as the molecular composition of the starch (Sadegizadeh-Yazdi *et al.*, 2019; Shah *et al.*, 2015). Modified thermoplastic blends can be used to package a variety of goods such as vegetables, fruits or perishable foods that spoil easily (Wesley *et al.*, 2014; Zhang *et al.*, 2019). Starch has also been used in many studies to produce stable biofilms (Lefatshe *et al.*, 2017) which have been used as a protective barrier in food packaging (Ugalde *et al.*, 2017; Fadeyibi *et al.*, 2017). In this study, starch was extracted from potato peels.

1.2.8 Previous studies using ZnO in food packaging

ZnO is an effective antibacterial agent important in food preservation. Mizielinska *et al.* used materials with polylysine or ZnO on cod fillets and found that the texture of the fish did not degenerate whilst the fish was packed in ZnO nanomaterials compared to the polylysine material (Mizielinska *et al.*, 2018). Furthermore, the cod also showed less water loss and decreased adhesiveness whilst the fish was packed in boxes for an extended amount of time (Mizielinska *et al.*, 2018). A study by Beak *et al.*, was able to synthesize a gelatin ZnO nanocomposite film for packaging spinach (Beak *et al.*, 2017). After incubation for some days, antimicrobial assays showed that the film was active against *L. monocytogenes* without affecting its quality or nutrient content (Beak *et al.*, 2017). Youssef *et al.* used carboxymethyl chitosan ZnO nanocomposite film on Egyptian soft

cheese, which improved its shelf life (Youssef *et al.*, 2016). The Egyptian cheese was incubated with the bio nanocomposite for several weeks and after evaluation, the sensory qualities and nutrient content were seen not to have significantly changed (Youssef *et al.*, 2016). Jafarzadeh *et al.*, investigated the effect of a ZnO nanocomposite with kaolin nanoclay and semolina flour on mozzarella cheese stored up to 72 days at low temperatures (refrigerated) (Jafarzadeh *et al.*, 2018). They found that the film had strong antimicrobial activity against food pathogens *E. coli*, *S. aureus*, *Candida albicans* and *Aspergillus niger*. The microbial growth was less than 2,5 CFU/g after incubation time and the quality was still acceptable compared to the cheese packaged in plain plastic packaging (Jafarzadeh *et al.*, 2018).

The feasibility of adding ZnO to polymeric nanocomposites for food packaging has been tested. For example, Li *et al.*, coated a polyvinyl chloride (PVC) film with ZnO NPs and reported antibacterial activity against *E. coli* and *S. aureus* (Li *et al.*, 2010). Li *et al.*, verified the potential of nano-packaging containing ZnO NPs in the storage process of Fuji apple slices and observed better preservation quality indicators, such as ascorbic acid, polyphenol content and lower typical changes in the number of microorganisms. (Li *et al.*, 2011). In addition, they found that at the end of storage, compared to PLA (polylactic acid) film, nanohybrid packaging film has the highest weight loss; however, packaging nanomaterials can better maintain firmness, color and sensory quality. It also shows a strong inhibitory effect on the growth of microorganisms (Li *et al.*, 2011). Fadeyibi *et al.*, synthesized a ZnO cassava nanocomposite which was used to package tomatoes at different temperatures and showed that the nanocomposite decreased microbial growth as well as retained the quality of the tomatoes (Fadeyibi *et al.*, 2017). Kumar *et al.*, synthesized a bio-nanocomposite film with ZnO NPs in agar using the solution casting method (Kumar *et al.*, 2019). The films were used to package green grapes, which were dried and ground, up to 25 days in ambient conditions. The grapes remained acceptable as spoilage was greatly decreased compared to the grapes packaged in normal plastic films (control) (Kumar *et al.*, 2019). These studies have proven to a larger extent that ZnO has desirable antibacterial and antifungal properties and can significantly prolong shelf life whilst reducing microbial spoilage.

1.3 Significance of study

Several treatments such as heat, synthetic chemicals and freezing have been used on perishable food to decrease the effects of spoilage. Some of these treatments are not as efficient in preservation, some chemicals cause toxic side effects whilst treatments such as freezing and heating alter the taste of the food (Bajpai *et al.*, 2018). A lot of foods are lost post-harvest due to spoilage by pathogens, hence there is a need to find natural and environmentally friendly methods that can be used to prolong their shelf life. Fresh fruits such as strawberries are known to spoil easily due to their intrinsic and extrinsic factors. They are easily contaminated due to their soft flesh and require proper storage to stay fresh for longer. In nanotechnology, NPs have been studied for their antifungal and antibacterial properties (Ortega *et al.*, 2017; Parashar *et al.*, 2020; Godymchuk *et al.*, 2015). These NPs can be immobilized in a matrix or polymer to restrict the NPs from depositing on the food. Immobilization of NPs, results in the packaging being biodegradable and safe for the environment. ZnO NCs have been proven to have excellent antimicrobial and antifungal properties, therefore can potentially be used in the food industry to package perishable foods to prolong their shelf life (Beak *et al.*, 2017; Fadeyibi *et al.*, 2017; Muthulakshmi *et al.*, 2017).

Currently, there is no nano-packaging available for prolonging the shelf life of strawberries as well as other fresh foods. Therefore, this study aims to develop a ZnO-starch NC paper and assess its antibacterial and antifungal properties as well as its efficacy in prolonging the shelf life of packaged strawberries. Adding the ZnO NPs into the starch-NC is expected to enhance the antibacterial properties of the ZnO-starch NC paper which in turn will slow down the spoilage of the packaged fruits. Developing and investigating the ZnO-starch NC paper is expected to pave the way for further investigation and use of NPs in the fresh food industry.

1.4 Aim and specific objectives

The main aim of this study was to investigate the antibacterial and antifungal properties of a ZnO-starch nanocomposite coated paper on strawberries to determine its efficacy in prolonging the shelf life of strawberry fruits.

To achieve the aim, the following specific objectives were undertaken:

- a) To synthesize and characterize starch, ZnO and ZnO-starch nanocomposite.

- b) To develop and examine the ZnO-starch nanocomposite paper for antifungal and antibacterial activity.
- c) To determine the efficacy of the coated paper in prolonging the shelf life of strawberries.

Chapter 2: Materials and Methods

2.1 Materials

The strawberry fruits were purchased from the local market and sterilized by submersion in sodium hypochlorite solution for 15 minutes at room temperature followed by air drying to remove any remaining surface liquid. The fruits were individually wrapped in wrapping paper and stored in zip lock bags at 4°C until further analysis.

2.2 Synthesis of starch, ZnO and ZnO-starch nanocomposite

2.2.1 Preparation of potato peel starch

Fresh potato peels were obtained from tuckshops around Palapye and stored at 4°C before starch extraction. The potato peels were first sorted and washed to remove dirt and any unwanted materials. After draining, the potato peels were pulverized into a paste using a blender and distilled water. The resultant paste was placed into a large container and stirred vigorously for 10 minutes to ensure the release of starch from the peels. The potato peels-starch solution was then strained through two layers of mutton cloth to separate the potato pulp from the starch slurry. After removing the pulp completely, the starch slurry was left at room temperature for 24 hours to allow the starch to settle. The top liquid solution was decanted to leave the starch at the bottom, washed several times with distilled water and centrifuged at 4000 x g for 5 minutes until a clear white starch solution was obtained. The starch was oven-dried at 60°C overnight to obtain a white powder. Figure 2.0 below outlines the extraction process.

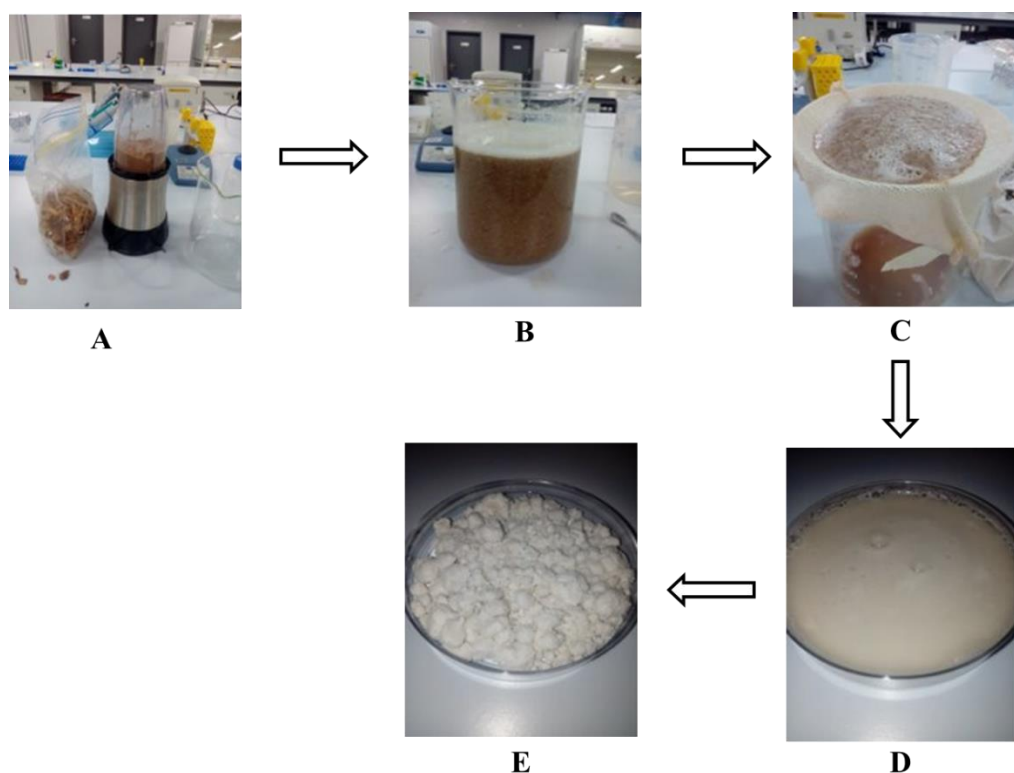
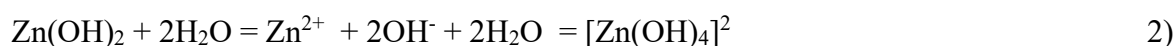
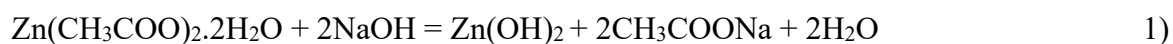


Figure 2.0. Extraction of potato starch from peels. A) potato peels pulverized into a paste, B) potato paste, C) draining through mutton cloth, D) potato starch after centrifuging and E) dried potato starch.

2.2.2 Synthesis of ZnO nano structures

ZnO NPs were synthesized using the hydrothermal method with zinc acetate dihydrate ($\text{Zn}(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$) (Sigma Aldrich, Germany) as the source of zinc and sodium hydroxide (NaOH) (Sigma Aldrich, Germany) as the reducing agent (Equation 1 – 4). For the ZnO NPs, 10 g of $\text{Zn}(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$ was dissolved into 200 ml of distilled water whilst 2.7 g of NaOH was dissolved into 200 ml of distilled water. The two solutions were mixed with 5 ml of Polyethylene glycol (PEG) (surfactant). After mixing the solutions thoroughly, they were transferred to a 500 ml media bottle and placed in the SA-252F Sturdy Apex group hydrothermal autoclave at 121°C for 2 hours until a white precipitate formed. The precipitate was washed with distilled water several times and oven-dried overnight at 65°C to obtain a white powder. The white powder was then calcinated by placing the powder at 200°C for 2 hours in the oven. Figure 2.1 shows the production process.



10 g of $\text{Zn}(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$ was dissolved in 200 ml of dH_2O whilst 2.7 g of NaOH was dissolved in 200 ml of dH_2O . The $\text{Zn}(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$ and NaOH solutions were mixed together.



About 5 ml of Poly ethyl glycol was added to the mixture and stirred for 5 minutes with a magnetic stirrer. Mixture was transferred to a media bottle and placed in the hydrothermal autoclave (121°C for 2 hours).



Product was washed and filtered 5 times with distilled water to obtain ZnO powder. Powder was dried at 60°C to obtain ZnO powder.



ZnO powder was calcinated at 200°C for 2 hours in oven to remove any impurities and converting all $\text{Zn}(\text{OH})_2$ to ZnO.

Figure 2.1. Flow process of producing ZnO powder using the hydrothermal method.

2.2.3 Preparation of ZnO starch nanocomposite

For the ZnO-starch NCs, 10%, 15%, 18% and 20% w/w zinc to starch mixtures were prepared by adding 15 g of potato starch to each of the samples. In a typical cycle, zinc acetate dihydrate was dissolved in 200 ml of distilled water and added to equivalent sodium hydroxide solutions for each concentration of NC (Table 1). The same conditions as in the synthesis of the ZnO NPs (Figure 2.1) were observed for each sample. After mixing, 15g of starch was added and the resultant mixtures were placed in 500 ml media bottles and placed in the hydrothermal autoclave at 121°C for 2 hours. The resultant white precipitate was filtered off and washed several times with deionized water to remove any by-products. The white filtrate was then dried in an oven at 65°C for 24 hours. After drying, the ZnO-starch

NC was annealed at 200°C for 2 hours and the resultant materials were ground into a powder and kept at 4°C in airtight containers.

Table 1. Amount of Zn(CH₃COO)₂·2H₂O and NaOH used to synthesize different concentrations of NCs.

NCs	Amount of Zn(CH ₃ COO) ₂ ·2H ₂ O (g)	Amount of NaOH (g)
10%	4.046	1.475
15%	6.069	2.212
18%	7.283	2.654
20%	8.092	2.949

2.3 Characterization of ZnO, potato starch and ZnO-starch nanocomposite

2.3.1 X-ray Diffraction

The ZnO powder, potato starch and ZnO-starch NCs were characterized using the powder X-ray diffractometer (XRD) (D8 Advance diffractometer, Bruker Germany) using Cu K α radiation of wavelength $\lambda = 0.1541$ nm in the scan range $2\theta = 20-90^\circ$. This machine gives information on the composition and nature of the bonding materials within the NPs and NCs. The crystallite size of the NPs was calculated using the Scherrer formula (Equation 5).

$$D = \frac{0.9\lambda}{\beta \cos\theta} \quad 5)$$

Where D is the crystallite size, λ is the wavelength of the X-ray (0.1541 nm), β is the full width at half maximum and θ is the Braggs angle.

2.3.2 UV-Vis spectrophotometry

The optical transmission/absorption spectra of ZnO, potato starch and ZnO-starch NC dispersed in water was recorded using the UV-Vis spectrophotometer (Lambda 750 Perkin Elmer UV-Vis-NIR spectrophotometer, Sydney, Australia) between the wavelength ranges of 200 - 1000 nm.

2.4. Determination of antibacterial and antifungal activity of ZnO-starch NC

2.4.1 Antifungal activity

The antifungal properties of the ZnO-starch NC were tested on Sabouraud dextrose agar (SDA) (Sigma Aldrich, Germany) by dissolving 31 g of the agar powder in 500 ml of distilled water in a 500 ml media bottle. After autoclaving, the media was left to cool to about 40°C and 1 ml of the antibiotic, streptomycin sulfate with a concentration of 0.1 g/ml was added to the media. The media was poured into petri dishes and left to solidify in the fume cabinet for 30 minutes. The sterilized strawberries were cut into small 1 cm cubes and placed on the SDA agar and left at room temperature for 5 days until fungi had grown on the agar. The fungi *B. cinerea* was then isolated according to morphological methods according to their phenotypic characteristics such as appearance and color (Figure 2.2A). The conidia and conidiophores of the fungi were observed under the microscope (Figure 2.2B and C) according to previous findings (Crous *et al.*, 2009, Tanovic *et al.*, 2009; Latorre *et al.*, 2015). The fungi were also identified by the color and appearance of the Apothecia (Figure 2.2D). The fungi were sub-cultured further into new SDA media plates and left at room temperature to grow for 5 days to obtain a pure culture. The antifungal disk diffusion method was used to test the effect of the ZnO-starch NC on the fungi. Different concentrations were made with each of the NCs by mixing them with sterilized distilled water to get concentrations of 0.1 g/ml, 0.2 g/ml and 0.3 g/ml. Round disks of about 8 mm in diameter were made from Whatman filter paper and sterilized in an autoclave. The disks were impregnated with 10 µl of the ZnO-starch nanocomposite solutions and left to dry in an oven at 60°C for 30 minutes. The antifungal cycloheximide (0.01 g/ml) and ZnO (0.1 g/ml) were both used as the positive controls whilst the potato starch (0.1 g/ml) was used as the negative control. *B. cinerea* was inoculated on the SDA media plates and the impregnated disks of the NCs and the controls were placed on the agar and the plates were placed at room temperature for 5 days.

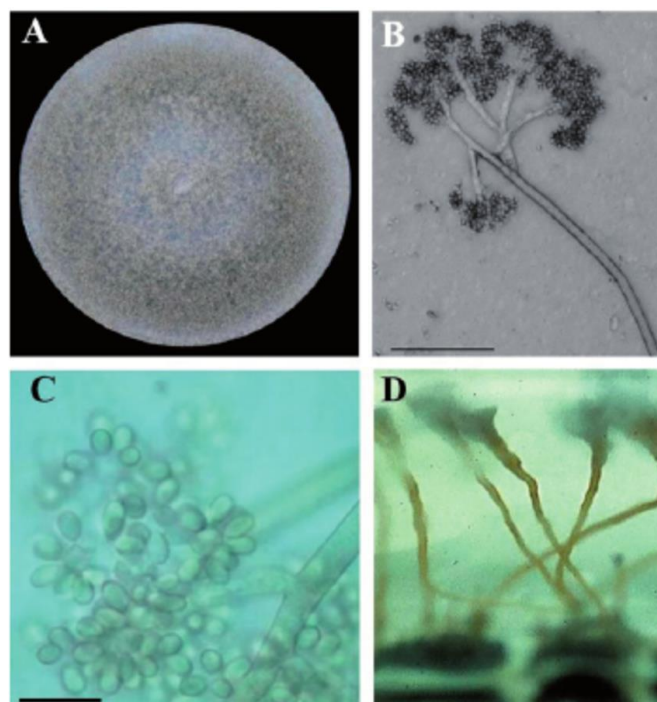


Figure 2.2. Morphological characteristics of *Botrytis cinerea*. A) Morphology on potato dextrose agar B) Conidiophore C) Conidia D) Apothecia (extracted from Latorre, *et al.*, 2015).

2.4.2 Antibacterial activity

The antibacterial properties of the NCs were tested on Mueller Hinton agar (MHA) (Sigma Aldrich, Germany) by dissolving 19.5 g of agar powder into 500 ml of distilled water in a 500 ml media bottle. After autoclaving and cooling, 1 ml of the antifungal cycloheximide at a concentration of 0.01 g/ml was added to the media. Bacterial cultures of *E. coli*, *P. aeruginosa*, *B. subtilis* and *S. aureus* were grown in nutrient broth at 37°C with shaking overnight. On MHA agar, 100 µl of the bacterial suspension was inoculated using the spread plate method. The positive controls for the antibacterial assay were Streptomycin sulfate which was at a concentration of 0.1 g/ml and pure ZnO NPs (0.1 mg/ml) as they have been proven to have antibacterial/antifungal properties in previous studies (Kumar *et al.*, 2019; Mizielinska *et al.*, 2018), whilst the negative control was the potato starch solution (0.1 g/ml). The impregnated disks were transferred to the inoculated plates which were incubated at 37°C for 24 hours. The assays were done in triplicates. After incubation, the plates were analyzed for inhibition zones around the inoculated disks and measurements taken.

2.4.3 Investigation of antibacterial and antifungal activity of ZnO-starch nanocomposite paper on strawberries

To make the ZnO-starch NC paper, the concentration of NC with the best results in the antifungal and antibacterial assay was chosen (0.2 g/ml). The NC was diluted with sterilized distilled water and kept at room temperature overnight after which it was homogenized in a blender until a smooth paste was obtained. Whatman filter paper was autoclaved and dipped into the NC solution. After the paper was fully saturated, it was placed on aluminum foil and placed in an oven at 70°C for 1 hour until completely dry (Figure 2.3). Plastic containers made from clear High-Density Poly Ethylene (HDPE) plastic were purchased from a local store. They were washed and sprayed with 70% alcohol to kill any pathogens present in the containers. After drying the containers at 60°C, the impregnated filter papers were trimmed and glued inside the plastic containers. The glue was left to dry completely after which a sterile paper towel was placed at the bottom of the container to suck up any moisture or liquid that may appear during the experiment. Sterile strawberries were inoculated with 1 ml of bacteria (*P. aeruginosa*, *B. subtilis*, *E. coli* and *S. aureus*) and left to completely dry at 40°C. *B. cinerea* was transferred onto the fruits using a sterile toothpick. The strawberries were placed in containers with the ZnO-starch NC paper (Figure 2.4A-D), while the plastic containers without the ZnO-starch NC paper were used as the positive control (Figure 2.4E). Each container had strawberries inoculated with a single bacterium type along with its positive control. The conditions of the experiment were kept constant for all the containers. The experiment had 2 sets of containers, one was placed at room temperature and the other set was placed at 4°C for a period of 10 days. The progress of the experiments was documented by taking pictures every 5 days to be compared at the end of the experiment.

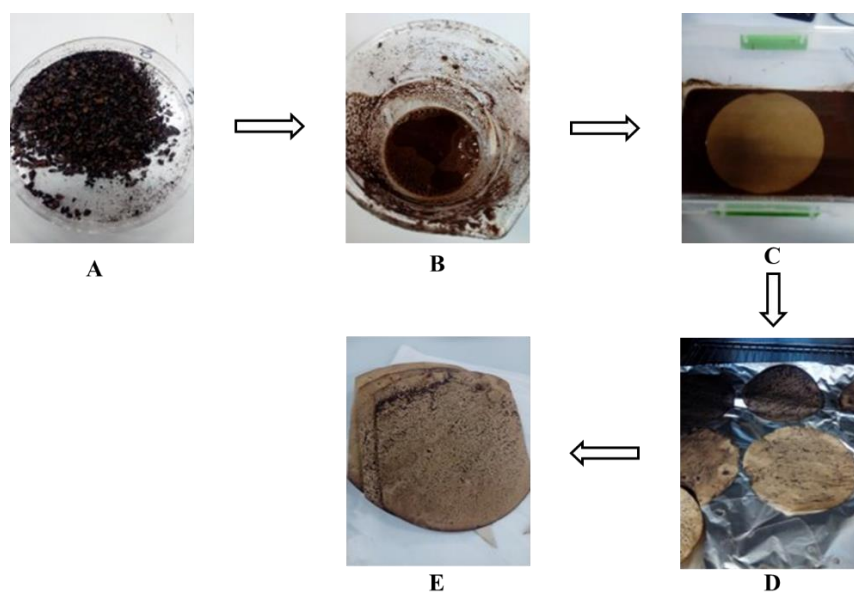


Figure 2.3. Preparation of the ZnO-starch NC paper. A) ZnO-starch NC, B) ZnO-starch NC paste, C) sterile filter paper dipped in NC paste, D) oven dried ZnO-starch NC paper and E) ZnO-starch NC paper trimmed to fit plastic packaging.

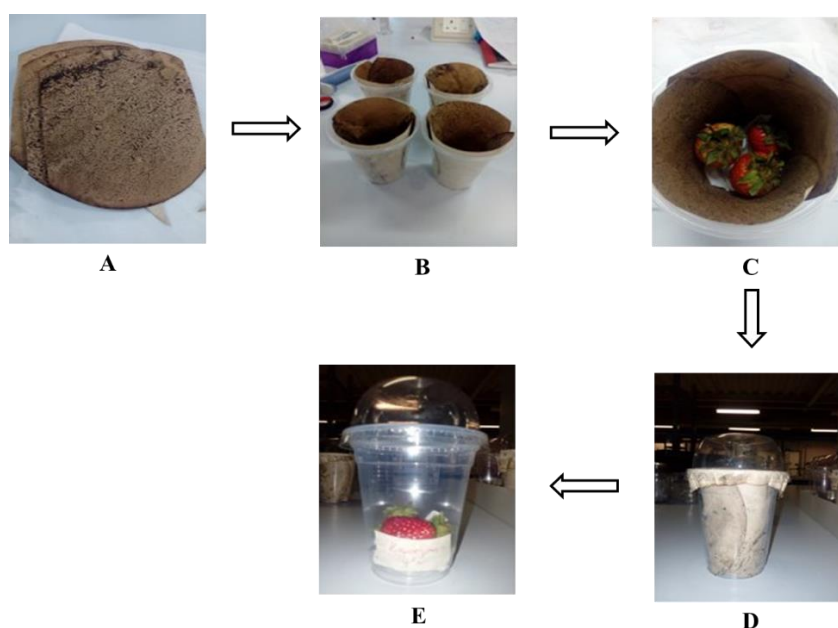


Figure 2.4. Packaging for strawberries with ZnO-starch NC paper and controls. A) trimmed ZnO-starch NC paper, B) plastic containers with the ZnO-starch NC paper, C) inoculated strawberries placed on the paper towel, D) strawberry packaging with ZnO-starch NC paper, sterile paper and inoculated strawberries and E) controls with paper towels, inoculated strawberries and without ZnO-starch NC paper.

2.5 Antifungal and antibacterial activity of ZnO-starch NC paper

2.5.1 Antifungal activity

The antifungal activity of the synthesized ZnO-starch NC paper was determined using the spread plate method. *B. cinerea* was spread using an inoculation loop on SDA agar with Streptomycin sulfate as an antibiotic. The ZnO-starch NC paper was cut into small 1 by 1 cm squares and firmly placed on the agar. A 1 cm by 1 cm square of plain sterilized filter paper was used as the negative control whilst a disk impregnated with the antifungal cycloheximide was used as the positive control. The plates were placed at room temperature for 5 days after which measurements for inhibition zones were taken.

2.5.2 Antibacterial activity

The antibacterial activity was determined by using the spread plate method. The bacterial cultures of *E. coli*, *P. aeruginosa*, *S. aureus* and *B. subtilis* (100 µl) were spread plated on MHA with the antifungal cycloheximide. A 1 by 1 cm square of the ZnO-starch NC paper was placed on the plates as well as the plain sterilized filter paper as the negative control and a disk impregnated with the antibacterial Streptomycin sulfate was used as the positive control. The plates were placed at 37°C for 24 hours after which the plates were analyzed for inhibition zones.

2.6 Quality assays on samples

The following assays were performed on the samples before incubation as well as after incubation.

2.6.1 Measurement of pH

The pH of fruits is determined by acids and other compounds. During storage, the fruit continues to respire and microbes growing on the fruit use up nutrients such as Vitamin C resulting in an increase in pH resulting in a change of sensory parameters. Hence pH measurement shows the extent of nutrient depletion and decreases in sensory quality in the fruit during incubation. The pH of the strawberries was tested using a standard portable pH meter. The fruit was homogenized in a blender to obtain a smooth slurry. The slurry was diluted 10 times with distilled water and centrifuged at 4000 x g for 5 minutes and the clear liquid was used for analysis.

2.6.2 Weight loss

During storage, fruits lose moisture and nutrients through respiration and microbial decomposition. The ZnO-starch NC paper is expected to decrease the amount of moisture and nutrients lost during the experiment hence this measurement is done to compare the weight before and after incubation. Weight loss was determined by weighing the strawberries before and after the experiment using a standard weight scale. The results were calculated as percentage weight loss according to Equation 6.

$$\text{Weight loss (\%)} = \frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \times 100 \quad 6)$$

2.6.3 Vitamin C content

Vitamin C content is essential for maintaining anthocyanins in the fruits which are responsible for the vibrant color of the strawberries. With time, Vitamin C contents reduce due to polymerization, condensation, microbial depletion and oxidation hence their measurement shows the effectiveness of the ZnO-starch NC paper in decreasing microbial growth and storage-related nutrient loss. Total Vitamin C content in strawberries was determined using the titration method. About 6.78 g of strawberry slurry was diluted 10 times with distilled water. The mixture was centrifuged at 4000 x g for 5 minutes to obtain a clear supernatant. The supernatant was transferred to a 250 ml Erlenmeyer flask. A 0.0322M iodine solution was used to determine the amount of Vitamin C in the strawberry juice. The iodine was poured into the burette and the final reading was noted. A 0.1% starch solution was prepared by dissolving 100 mg of soluble starch into 10 ml of distilled water. The starch solution was used as an indicator by adding a few drops into the strawberry juice. The starch solution reacts with the iodine solution to form a blue-black complex after all the Vitamin C in the supernatant has reacted with the Iodine, denoting the endpoint. The strawberry juice was titrated against the iodine solution until a constant blue-black color that persisted for more than 30 seconds was observed. The final reading of the iodine solution was noted. The titration procedure was done in triplicates and the average was obtained to calculate the amount of Vitamin C present in the strawberry juice using Equation 7.

$$\text{Vitamin C} = [\text{Iodine used (L)} \times [\text{Iodine}](\text{M})] \times \text{MW of Vit C (176.14 g)} \quad 7)$$

2.6.4 Total phenolic content

Phenolic compounds are responsible for maintaining the sensory quality of the fruits. With storage, the phenolic compounds become depleted and are targeted by enzymes as well as microbes growing on the fruits. To ascertain if the ZnO-starch NC paper is effective in food preservation, measurement of the phenolic compounds in strawberries is taken and compared to the normal range of phenolic compounds found in the strawberry fruits. The total phenolic content was determined using UV spectrophotometry. The oxidizing reagent Folin–Ciocalteu reagent (FCR) was utilized. A stock solution for gallic acid was prepared by dissolving 500 mg of gallic acid powder in 10 ml of ethanol and then diluted to 100 ml with distilled water to obtain a stock solution with a concentration of 5 g/L. A series of 5 gallic acid standards were then prepared by adding 1, 2, 3, 5 and 10 ml of the stock solution, respectively to a 100 ml volumetric flask and diluted to volume with sterilized distilled water. These were to make standard solutions of concentrations 50, 100, 150, 250 and 500 mg/L which were then used to plot a calibration curve to estimate the number of phenolic compounds in the fruits. A 200 g/L Sodium carbonate (Na_2CO_3) solution was prepared by dissolving 20 g of anhydrous Na_2CO_3 powder in 50 ml of distilled water. The mixture was boiled on a heating block until all the powder was dissolved and the solution was left to cool overnight. The cooled Na_2CO_3 mixture was diluted to 100 ml with distilled water.

For the phenolic compound assay, a series of 9 test tubes were set up, 5 for the gallic acid standards, 1 for the blank and 3 for each of the strawberry juice test samples. In each test tube (1 – 9), 15 ml of distilled water and 1ml of the FCR were added. In tubes 2 – 6, 1 ml of the gallic acid standards were added, respectively, whilst in tubes 7 – 9, 1 ml of the test sample was added (assay was done in triplicates). For the blank 1 ml of distilled water was added. All the tubes were left at room temperature to incubate for 6 minutes. After incubation, 3 ml of Na_2CO_3 was added to each test tube and the tubes were placed in a water bath at 40°C for 30 minutes. One ml of the mixture was added to cuvettes and absorbance was read at the 765 nm wavelength. The absorbance of the gallic acid standards were used to plot a calibration curve. The calibration curve was used to calculate the total phenol content using Equation 8.

$$\text{Total phenolic } \left(\% \frac{w}{w} \right) = \frac{A-b}{m} \times \frac{V D}{W \times 1000} \times 100 \quad 8)$$

Where A is the absorbance of the sample test solution, b is the y intercept of the calibration curve, m is the slope of the calibration curve, W is the weight of the test material (mg), V is the volume of the sample test solution and D is the dilution factor.

2.6.5 Analysis of the effect of ZnO-starch NC paper against bacterial growth

After the 10-day incubation of the strawberries, the amount of bacterial growth was assayed using the spread plate method whilst the amount of fungal growth was assayed using the pour plate method. A 0.9% saline solution was prepared by dissolving 4.5 g of sodium chloride in 500 ml of sterile distilled water. Strawberries from each container were crushed using a pestle and mortar. About 1 g of the fruit was measured and placed in a sterile test tube and 9 ml of saline solution was added to it to make a 10^{-1} bacterial/fungal suspension. This suspension was used to make two more serial dilutions in saline solution up to a suspension of 10^{-3} dilution. For the bacterial count, 100 μ l of the bacterial suspensions were inoculated on MHA agar with two sets of plates, one for strawberries incubated with the NC and the other set for the strawberries incubated without the nanocomposite (positive control). The plates were placed at 37°C for 24 hours followed by counting of the colonies. The colony forming units per Gram (CFU/g) were calculated (Equation 9).

$$\frac{\text{CFU}}{\text{g}} = \frac{\text{Number of CFU/ml}}{\text{g/ml}} \quad 9)$$

For the fungal count, 1 ml of the fungal suspensions was transferred to empty sterile petri dishes. Liquid SDA agar with the antibiotic Streptomycin sulfate was prepared and kept liquid in a 50°C oven. The agar was poured into the petri dishes and mixed with the fungal suspensions by gently rotating the dishes. The agar was left to solidify at room temperature and incubated at 27°C for 3 days after which the formed fungal colonies were counted. Equation 10 was used to calculate the CFU/g.

$$\frac{\text{CFU}}{\text{g}} = \frac{C}{(a+0.1b+0.01c)d} \quad 10)$$

Where C is the sum of fungal colonies counted on the plates, a is the number of dishes retained in the first dilution resulting in fungal colonies between 10 and 150 colonies, b is the number of dishes retained in the second dilution resulting in fungal colonies between 10 and 150 colonies,

c is the number of dishes retained in the third dilution resulting in fungal colonies between 10 and 150 colonies and d is the dilution factor of the first dilution.

2.7 Statistical analysis

All the collected data were calculated as the mean of triplicate measurements. Microsoft Excel was used to conduct a one tail t-test with $p = 0.05$ to calculate if there was a significant difference between the fruits incubated with the NC paper and those incubated without the NC paper (controls). The data on the effect of the ZnO-starch NC paper on the strawberries (Microbial count) (Table 8) and weight loss % (Table 5) was analysed using a one tail t-test with a p value of 0.05. The test was done using Microsoft Excel 2010 with two hypotheses for both data sets, the alternative hypothesis stating that there is not any significant difference between the two groups and the null hypothesis stating that there is a significant difference between the two groups.

Chapter 3: Results and Discussion

3.1 Extraction and characterization of starch from potato peels

The potato peel contains about 66% starch (dry weight) and it would be more economic than using actual potatoes to extract starch (Nischala, 2019). The starch was successfully extracted as it is not soluble in water hence it settles to the bottom of the container due to its compact semi-crystalline structure made up of amylopectin and amylose layers (Bertoft, 2017). To test if the powder was indeed starch, a standard Iodine test was done, and a blue-black color was seen denoting that starch was present in the powder.

From the XRD analysis, the diffractogram of the potato starch (Figure 3.0A) showed that the strongest peaks were observed at the 17° position, medium peaks at the 15°, 22° and 24° positions whilst the weakest points were around the 10° and 19° 2 θ position. These positions show typical peaks with XRD diffractograms of starch from potato peels (Yan *et al.*, 2011; Shah *et al.*, 2015; Bertoft, 2017). Starch is known to contain 2 types of polysaccharide components namely branched amylopectin and linear amylose (Robyt, 2008; Bertoft, 2017). Amylose is very unstable in water therefore amylose content in the starch determines the properties of starch. Starch has 3 types of crystalline structures documented in previous studies, that is A-type (Duc and Do, 2019), B type (Shah *et al.*, 2015) and C-type structures (Cornejo-Ramírez *et al.*, 2018). Potato starch has the B-type crystalline structure which is exhibited by most plant tubers (Bertoft, 2017; Shah *et al.*, 2015). According to the XRD diffractogram, the synthesized potato starch has a B-type crystalline structure which is supported by assayed potato starch reported in literature (Bertoft, 2017; Shah *et al.*, 2015). The absorption spectrum of potato starch by UV-Vis spectrophotometry (Figure 3.1A), shows an absorption peak around the 630 nm wavelength. According to literature, most natural starches absorb light around the 610 nm position (Wuttisela, *et al.*, 2008; Fiedorowicz, *et al.*, 2010a; Fiedorowicz, *et al.*, 2010b). The results are similar to that of previous studies, but the shift to 630 nm could be due to the amylose content of the potatoes used which affects the crystalline structure of potatoes hence their absorption spectra (Robyt, 2008; Bertoft, 2017).

3.2 Synthesis and characterization of ZnO NPs and ZnO-starch nanocomposite

The ZnO-starch nanocomposites were synthesized in increments from 10% to 20% to get the ZnO to starch ratio which has the most effective antibacterial and antifungal activity as

ZnO NPs have been found to be highly effective at low concentrations (Bankier *et al.*, 2019; Liu *et al.*, 2009).

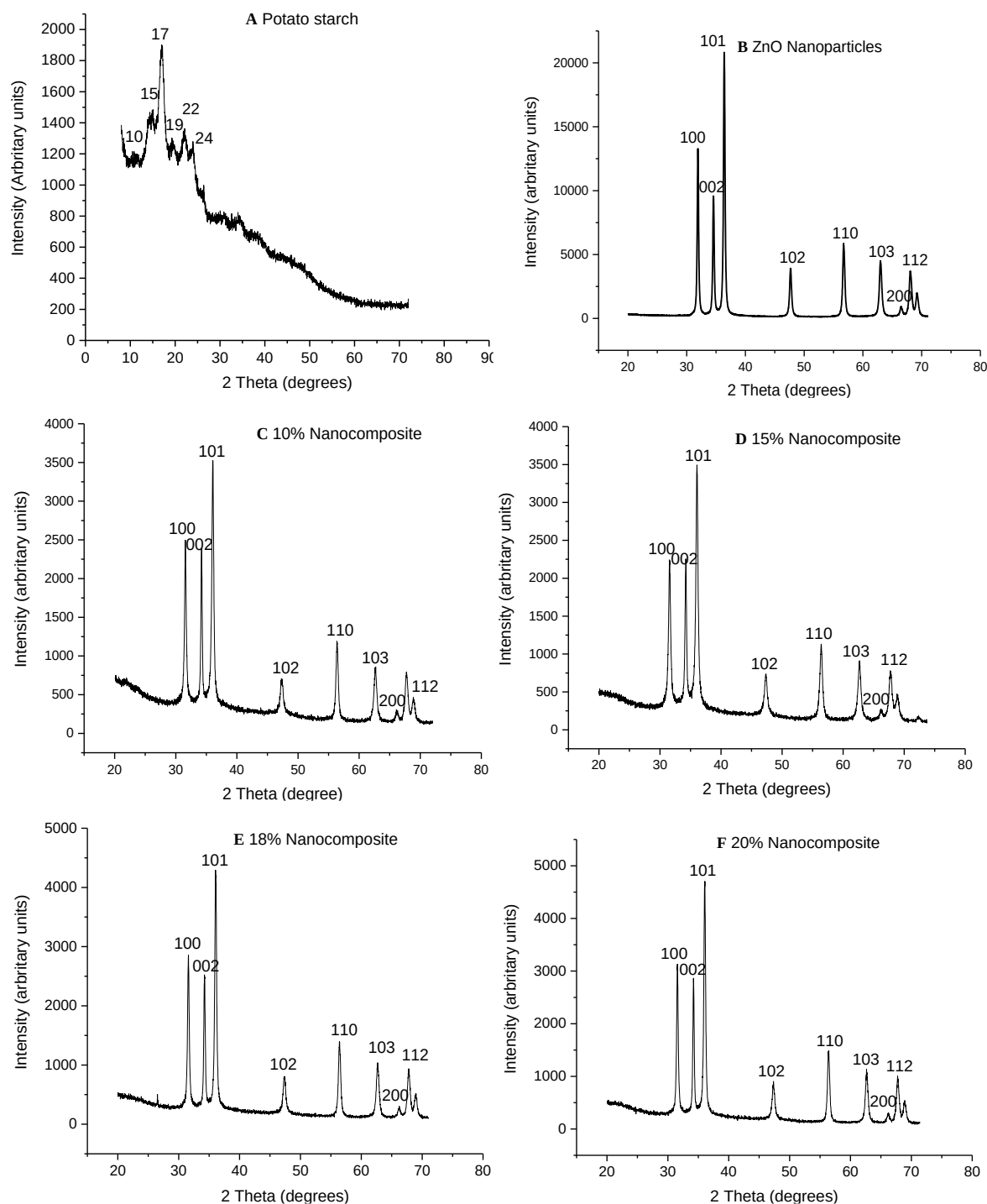


Figure 3.0. X-ray diffractogram profiles. A) Potato starch, B) ZnO NPs, C) 10% ZnO NC, D) 15% ZnO-starch NC, E) 18% ZnO-starch NC and F) 20% ZnO-starch NC.

The experiment started off with increments from 2% to 8% ZnO content according to previous studies (Fadeyibi *et al.*, 2016; Li *et al.*, 2011) but the results showed little to no antibacterial or antifungal activity hence the percentages were increased.

3.2.1 Characterization of synthesized ZnO and ZnO-starch NCs.

The chemical composition and crystallographic structure of the ZnO NPs, ZnO-starch NC and potato starch were measured by X-ray diffractometer and the XRD profiles are shown in Figure 3.0.

The ZnO and ZnO-starch NC diffractograms exhibited the standard diffraction peaks at 31.84° , 34.52° , 36.33° , 47.63° , 56.71° , 62.96° , 68.13° , and 69.18° , which have been indexed as the standard peaks for the ZnO hexagonal wurtzite phase. The diffraction peaks also correspond to the 100, 002, 101, 102, 110, 103, 200 and 112 planes, respectively according to the JCPDS database card no. 00-036-451. The ZnO diffraction pattern (Figure 3.0B) was matched with the Crystallography Open Database (COD) entry number 2300112 (<https://www.crystallography.net/cod/2300112.html>). Using the Scherrer equation (Equation 5), the crystallographic size of the ZnO was estimated to be between 30 – 35 nm in size. No other peaks were seen on the diffractogram hence it was concluded that the ZnO NPs were pure and no impurities were present. The ZnO-starch NC profiles (Figure 3.0C-F) showed a decrease in intensity of all the peaks compared to the ZnO NPs diffractogram. This was attributed to the percentage of ZnO precursor used in the synthesis of the ZnO-starch NC compared to the amount used for the synthesis of pure ZnO NPs. The peaks were all present at the same positions as the peaks of the bare ZnO NPs and the presence of the potato starch had not altered or changed the crystalline structure of the ZnO NPs. The graphs showed the presence of two phases attributed to the presence of both the ZnO NPs and the potato starch.

3.2.2 UV/Vis spectrophotometry

The optical properties and absorption spectra of the ZnO NPs and the ZnO-starch NC were measured using UV-Vis spectrophotometry (Figure 3.1). Knowing the absorption capabilities of a nanomaterial is essential in order to determine its correct application (Wang *et al.*, 2018). UV-Vis spectrophotometry method is used to measure how much light is absorbed and scattered by a material (Modena *et al.*, 2019). ZnO is a semiconductor and has very desirable electrical and photocatalytic properties. The absorption spectra of ZnO NPs (Figure 3.1B) shows an increased absorption peak at the 371 nm wavelength which is similar to the absorption peaks

reported before (Zak *et al.*, 2011; Mohsin *et al.*, 2021; Goh *et al.*, 2014; Farha *et al.*, 2020). The ZnO-starch NC absorbed light between the 248 and 258 nm wavelengths (Figure 3.1C-F).

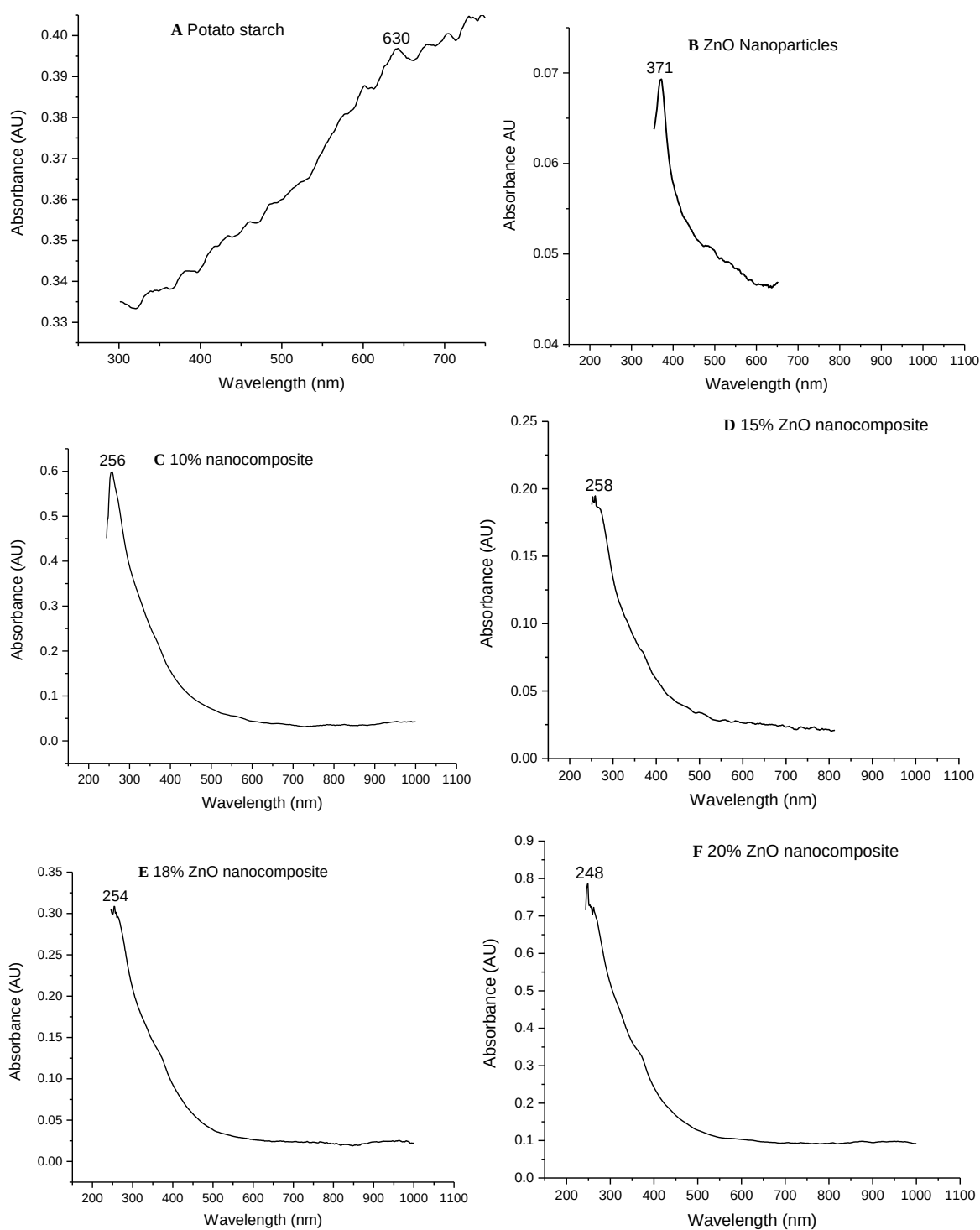


Figure 3.1. UV-Vis absorption spectrums. A) Potato starch, B) ZnO NPs, C) 10% ZnO NC, D) 15% ZnO-starch NC, E) 18% ZnO-starch NC and F) 20% ZnO-starch NC.

This means that the ZnO NPs and ZnO-starch NC absorb light in the UV region between 100 nm to 400 nm. The ZnO-starch NC had absorption peaks at a lower wavelength (248 – 258 nm) due to the presence of the potato starch which lowered the absorption capabilities of the NPs and the different concentrations of ZnO NPs added in each NC (Ma, *et al.*, 2016; Shankar, *et al.*, 2014). The antibacterial/antifungal properties due to photocatalysis can be improved or are at their highest if the ZnO is exposed to UV light at these wavelengths (Mazitova *et al.*, 2019; Smijs and Pavel, 2011).

3.3 Antibacterial and antifungal assay of ZnO- starch NC and ZnO-starch NC paper

The antibacterial/antifungal assay was done using the synthesized NCs against the selected pathogens *P. aeruginosa*, *B. subtilis*, *E. coli*, *S. aureus* and *B. cinerea* using the disk diffusion assay (Figure 3.2). The assay showed that *B. cinerea* along with the Gram-positive bacteria *S. aureus* and *B. subtilis* were susceptible to the ZnO-starch NCs whilst the Gram-negative bacteria *E. coli* showed minimal activity compared to the Gram-negative *P. aeruginosa* which was not susceptible. From the four ZnO-starch NCs synthesized (10%, 15%, 18%, 20%), the 15% ZnO-starch NC was chosen as having the highest antibacterial/antifungal activity. Different concentrations of the 15% ZnO-starch NCs were made (0.1, 0.2, 0.3 g/ml) to test for the best concentration to be used for subsequent experiments (Figure 3.3). The 0.2 g/ml concentration had the highest antibacterial/antifungal activity and was used in subsequent experiments.

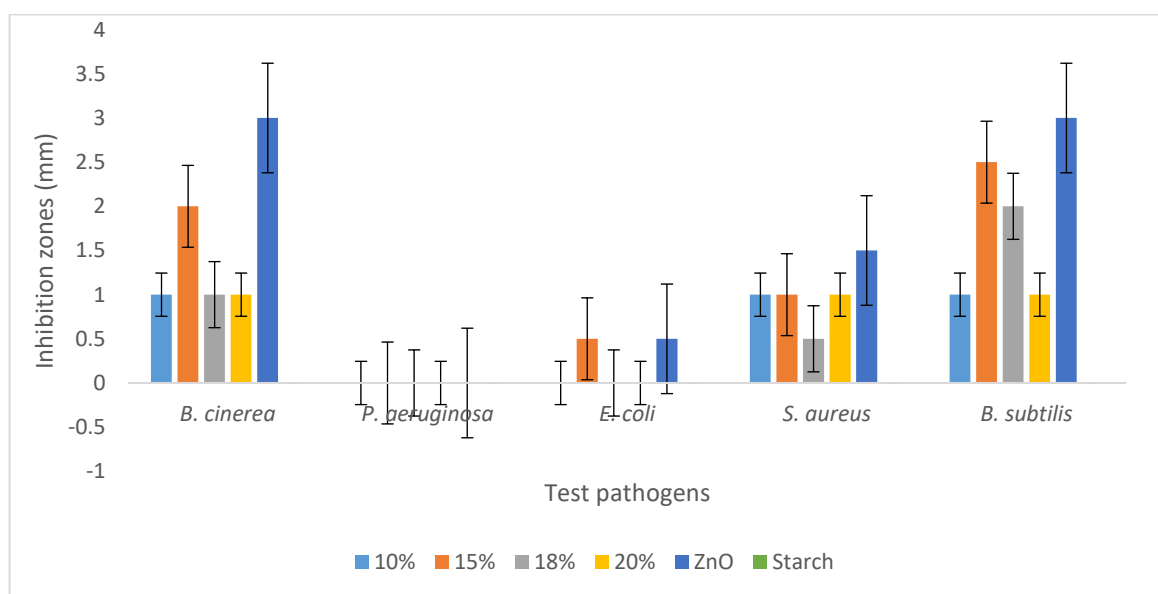


Figure 3.2. Size of inhibition zones for each nanomaterial against the respective test pathogens and controls.

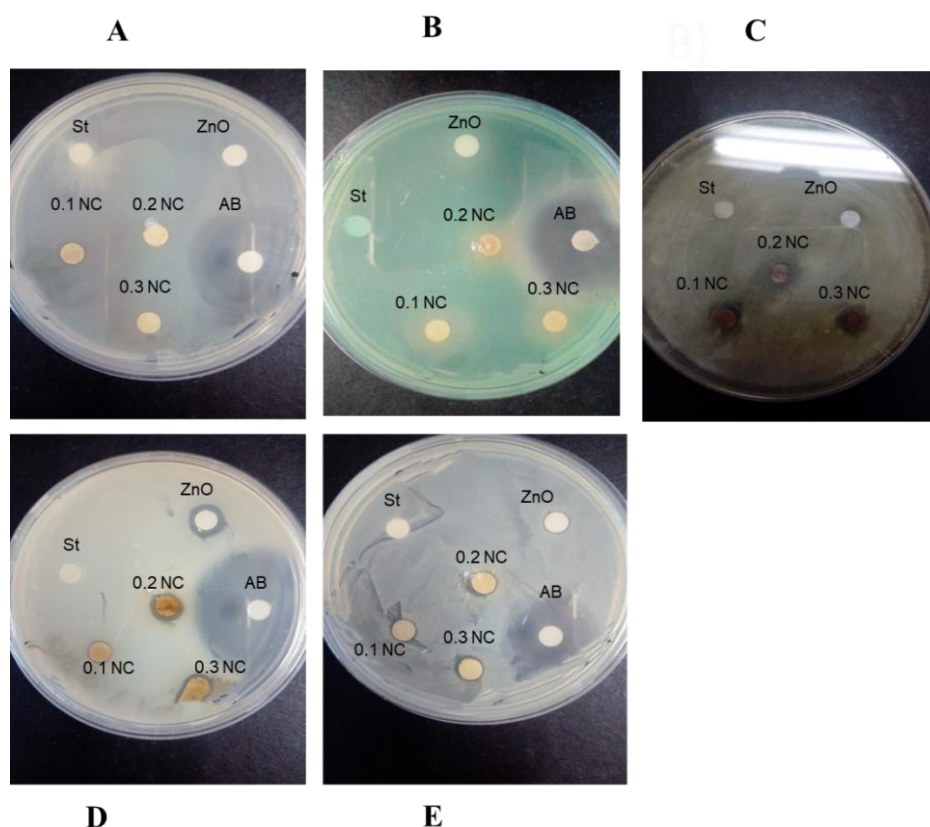


Figure 3.3. Results of the antifungal and antibacterial disk diffusion assay of the NC and the controls (ZnO and potato starch) against the test pathogens. A) *E. coli*, B) *P. aeruginosa*, C) *B. cinerea*, D) *B. subtilis* and E) *S. aureus*.²

The fungus *B. cinerea* was used as it is a very common spoilage pathogen that quickly affects strawberries and other fresh foods post-harvest (Jarvis, 2008; Feliziani and Romanazzi, 2016). The results show that the ZnO NC, were active against *B. cinerea*, *S. aureus* and *B. subtilis* but were not active against *P. aeruginosa* with minimal to no antibacterial activity against *E. coli*. The same antibacterial activity was observed for the control (pure ZnO NPs) whilst the negative control (starch) did not produce any inhibition for all the test pathogens. These were expected as starch has been proven not to have any antibacterial or antifungal properties (Ali *et al.*, 2021; Ramdath *et al.*, 2021). The ZnO NPs were observed to have a higher antibacterial and antifungal activity than the ZnO-starch NC since the Zn^{2+} ions produced by the ZnO NPs in the NCs tend

² AB = antibiotic, St = starch, AF = Antifungal, NC = nanocomposite.

to form complexes with starch in solution hence there are fewer metal ions available for antibacterial/ antifungal activity (Ma *et al.*, 2016).

The Gram-positive bacteria showed the most activity with *B. subtilis* having the highest recorded inhibition zones (2.5 mm). Both Gram-negative and Gram-positive bacteria have negatively charged cell walls, but Gram-negative bacteria have certain components like teichoic acids which make their cell wall to be more negative hence they are more susceptible to NPs. The difference in morphological structure of the bacteria can affect the antibacterial activity of the ZnO-starch NC (Slavin *et al.*, 2017; Neves *et al.*, 2019). Literature has reported that Gram-negative pathogens need a higher dosage of NPs than Gram-positive bacteria because of the presence of a cell wall and its components such as the lipopolysaccharides, which block the ZnO NPs from attacking the bacterial cells. This will result in a higher minimum inhibitory concentration (MIC) for Gram-negative than for Gram-positive bacteria (d'Água *et al.*, 2018; Tayel *et al.*, 2011; Reddy *et al.*, 2007). It has been reported before that bacteria such as *E. coli* are more susceptible to higher concentrations of ZnO as lower concentrations have only bacteriostatic effect whilst higher concentrations lead to cell death (Stanković *et al.*, 2013; Raghupathi *et al.*, 2011).

Smaller NPs of sizes below 12 nm have been reported to be more susceptible to Gram-positive bacteria than those of sizes above 30 nm, therefore, the sizes of the NPs are important for improved antibacterial activity (Lallo da Silva *et al.*, 2019; Sharma *et al.*, 2016). The synthesized ZnO NPs were about 35 nm which could have affected the ZnO-starch NC antibacterial activity as well as its MIC. The antibacterial assays showed that *S. aureus* was less susceptible to the NPs compared to the spore-forming bacteria *B. subtilis*. This could be attributed also to the ability of *S. aureus* to produce the compound Staphyloxanthin which is a carotenoid with antioxidant properties (Liu *et al.*, 2005; Clauditz *et al.*, 2006). This compound gives the bacteria protection against oxidative stress and ROS attack making *S. aureus* less sensitive to the NPs (Clauditz *et al.*, 2006). According to literature, ZnO has significant antifungal activity against *B. cinerea* and the results show that the ZnO NPs and ZnO-starch NC showed antifungal activity although the inhibition zones were unclear due to the overgrowth of the fungus (Mahovic *et al.*, 2005; He *et al.*, 2005).

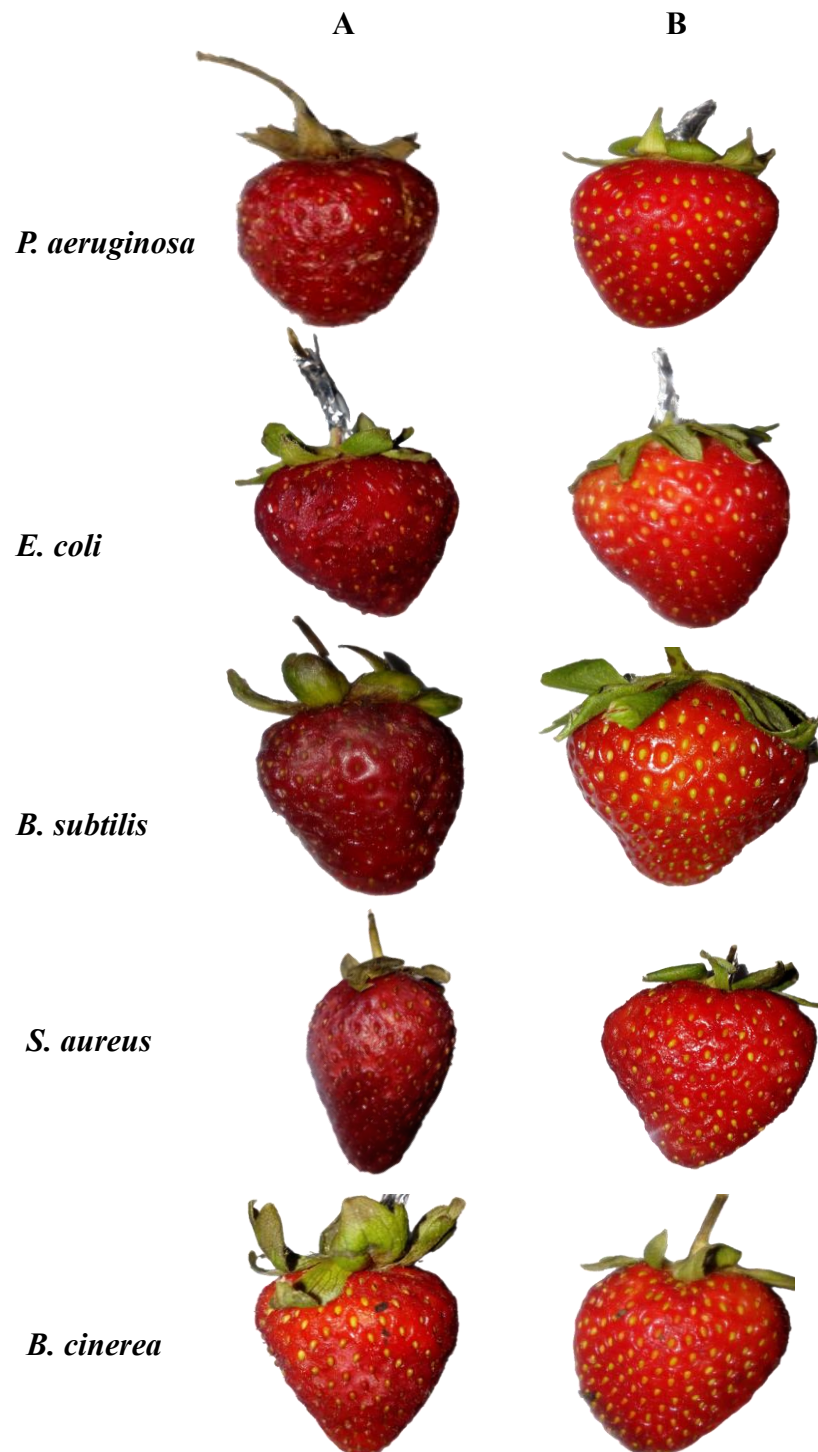


Figure 3.4. Strawberries inoculated with test pathogens kept at 4°C. A) Controls without ZnO-starch NC and B) with ZnO-starch NC paper.

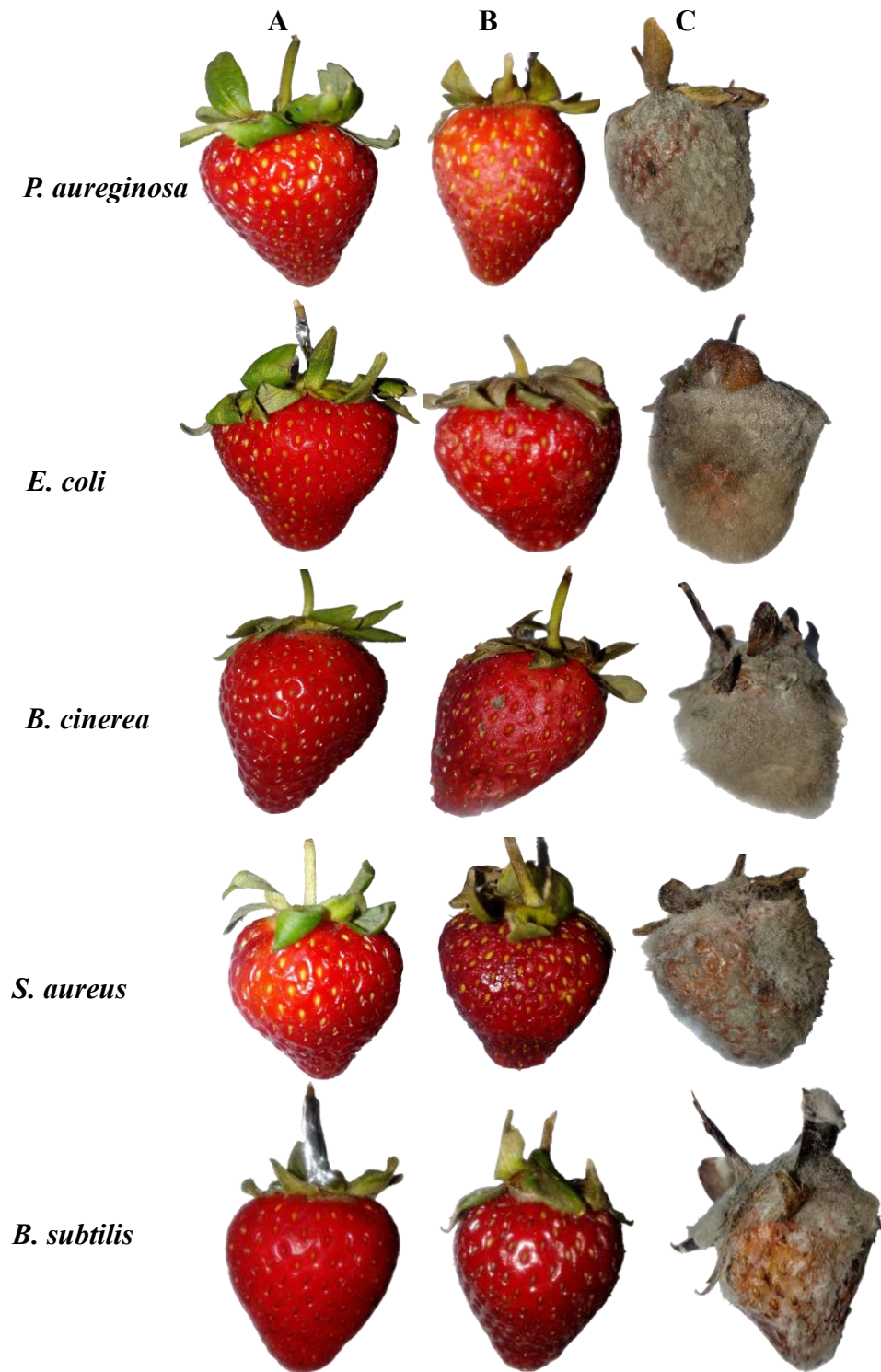


Figure 3.5. Strawberries inoculated with test pathogens incubated at 27°C with the ZnO-starch NC paper. A) Day 1, B) Day 5 and C) Day 10.

3.4 Antifungal and antibacterial activity of ZnO- starch NC paper on strawberries

The sterilized strawberries were first inoculated with bacteria (*P. aeruginosa*, *E. coli*, *B. subtilis* and *S. aureus*) and *B. cinerea* and observed for 10 days. The differences were observed between the strawberries incubated with the ZnO-starch NC paper and the controls for each pathogen. The experiment was done in 2 sets with one kept at 4°C (Figure 3.4) and the other kept at 27°C (Figure 3.5) to test the ability of the NC paper to keep the fruits fresh for longer if not refrigerated.

For the strawberries kept at 27°C, all the controls were completely rotten by the second day whilst the fruits incubated with the ZnO-starch NC paper were without mold until the 6th day and were completely spoilt by the 10th day hence quality parameters of the fruits were not measured. The strawberries kept at 4°C with the ZnO-starch NC paper were still viable, and the appearance was still acceptable after 10 days compared to the controls which had already started to show signs of spoilage by day 10. Fruits incubated at 4°C (Figure 3.5) show a significant difference in spoilage between the test sample and the controls. The controls showed spoilage by soft rot and mold. The fruits had a darker red color due to the oxidation of anthocyanins which are responsible for the red color in strawberries (Patras, *et al.*, 2009). In the presence of oxygen and ascorbic acids, anthocyanins are broken down leading to the decolorization of the anthocyanins. This occurs over elongated time periods during storage (Patras, *et al.*, 2009; Holzwarth *et al.*, 2012). The fruits incubated with ZnO-starch NC paper still maintained the original red color from the first day due to the oxygen scavenging ability of the ZnO NPs and the decrease in pathogen mediated nutrient depletion which leads the decrease in anthocyanin content (Patras, *et al.*, 2009; Holzwarth *et al.*, 2012). The ZnO-starch NC treated fruits incubated at 4°C (Figure 3.5) did not show any visible spoilage or mold growing on them. Not much difference was seen in the rate of spoilage of the fruits incubated with the 5 test pathogens at 4°C.

The fruits incubated at 27°C show that by the 5th day, though incubated with the ZnO-starch NC, they had spoiled tremendously and were no longer acceptable for consumption (Figure 3.6). All the controls had completely spoiled by the 3rd day. This means that the strawberries can stay a maximum of 3 days without refrigeration but not for longer than that period. The ZnO-starch NC can reduce the rate of spoilage for a few days but is not able to prolong the shelf life of the strawberry fruits for longer at room temperature. The ZnO-starch NC can improve shelf life in conjunction with low temperatures and as strawberries are stored between 2-8°C in stores, the nanomaterial can potentially be used in their packaging to prolong shelf life.

3.6 Quality parameters

The quality parameters of the strawberries were analyzed before and after the experiment. The tests were done in triplicates and the average of the three tests was noted. The experiment was done on a total of 100 strawberries as 5 strawberries were treated with each microorganism. The quality parameters were noted as ranging from the smallest to the largest measurement. The quality assays for pH, total phenolic content and Vitamin C content were done after the experiment as the strawberry fruits had to be pulverised to conduct the assay and hence could not be used for the packaging experiment. The quality assays were done after the experiment and compared to the normal ranges in literature as an alternative.

The quality parameters were calculated to measure the effectiveness of the ZnO-starch NC paper on the preservation of the fruits incubated with the NC paper compared to those incubated without the ZnO-starch NC paper. During packaging, pathogens grow on the fruits and they use up nutrients in the fruit to multiply and grow (Barth *et al.*, 2010). As the pathogens proliferate and the fruit continues to respire, compounds such as phenolic compounds, vitamins, sugars, and water slowly diminish resulting in spoilage and decreased sensory parameters (Moral *et al.*, 2017; Dilbaghi and Sharma, 2007).

3.6.1 pH

The pH of the strawberries was measured after the experiment using a standard portable meter. The pH values were found to be between 3.4 and 5.1 for the fruits incubated with the ZnO-starch NC paper whilst for the fruits incubated without the ZnO-starch NC paper, pH was found to be between 3.3 to 5.1 (Table 2).

Table 2. The pH values of the fruits incubated at 4°C with the ZnO-starch NC paper and without the ZnO-starch NC paper (controls).

Pathogen used	pH (NC paper)	pH (controls)
<i>P. aeruginosa</i>	3.9	4.8
<i>E. coli</i>	4.5	4.2
<i>S. aureus</i>	4.2	3.3
<i>B. subtilis</i>	3.4	5.1
<i>B. cinerea</i>	5.1	3.7

The pH of the fruits incubated with the NC paper and their controls was measured to be between 3.4 – 4.5 and 3.3 – 4.2, respectively. Fresh strawberries have a pH between 3.2 – 4.5 and these results show that the strawberries still have a normal pH even after incubation (Giampieri *et al.*, 2012). The pH was expected to decrease significantly due to respiration and the use of acids by microbes to proliferate. This shows that the pH quality assay was not useful in measuring the extent of spoilage of the strawberry fruits.

3.6.2 Weight loss %

Before the experiment, all the strawberries were weighed to obtain their initial weight and after the experiment, the strawberries were again weighed to obtain the final weight in triplicates (Table 3). The weight loss of each strawberry was calculated as % weight loss using Equation 6. All the fruits kept at 27°C were completely spoilt at the end of the experiment hence their final weight (and other quality parameters) could not be measured.

Table 3. Measurement of % weight loss of strawberries kept at 4°C with the ZnO-starch NC paper and without the ZnO-starch NC paper (controls).

Pathogen used	%Weight loss (controls)	%Weight loss (NC paper)
<i>P. aeruginosa</i>	3.49	2.21
<i>E. coli</i>	3.88	2.98
<i>S. aureus</i>	1.21	0.74
<i>B. subtilis</i>	0.83	0.24
<i>B. cinerea</i>	0.72	0.55

Weight loss was calculated as a percentage of the initial weight compared to the final weight of the strawberries. This was done to calculate the loss of moisture and nutrients due to respiration and the growth of pathogens in the fruits. The highest weight loss was obtained from the fruits incubated without the ZnO-starch NC paper whilst the fruits incubated with the ZnO-starch NC paper showed a lesser weight loss percentage. Studies on the application of ZnO based NC coatings on fruits (strawberries and salak) showed that the nanocomposites decreased weight loss compared to the controls by preventing water loss by respiration and evaporation as well as a reduction in the decomposition of nutrients in the fruit (Marpaung *et al.*, 2015; Emamifar and Bavaresi, 2020). The weight loss was calculated to be about 3% after 12 days of incubation which is similar to the highest weight loss attained in this study (3.88%) (Marpaung *et al.*, 2015;

Emamifar and Bavaisi, 2020). This could be attributed to the presence of starch and ZnO NPs which act as a physical barrier preventing high moisture loss and the growth of pathogens on foods which lead to nutrient depletion increasing weight loss (Petriccione *et al.*, 2015; Velickova *et al.*, 2013). Fruits incubated with the test pathogen *E. coli* was seen to have the highest weight loss whilst those incubated with *B. subtilis* exhibited the lowest weight loss proving that the pathogen with the highest susceptibility will have a lesser effect on the fruit's quality parameters. The overall weight loss showed that the loss of moisture and nutrients was not statistically significant ($p > 0.05$) as there was a small change in the weight of the fruits.

3.6.3 Total phenolic content

The total phenolic content of the strawberries was quantified using the FCR method. First, a calibration curve was plotted using gallic acid standards of different concentrations (50, 100, 150, 250, 500 mg/L) (Figure 3.6). The calibration curve equation $y = 0.004x - 0.027$ and Equation 7 were then used to calculate the phenolic content of the strawberries. The total phenolic compounds of the strawberries after the experiment were found to be between 54 and 96 mg/100g (NC paper) and between 55 and 109 mg/100g for the controls (Table 4).

Table 4. The total phenolic compounds of strawberries measured after incubation at 4°C with ZnO-starch NC paper and without the ZnO-starch NC paper (controls).

Pathogen used	Total Phenolic content (mg/100g) (NC paper)	Total Phenolic content (mg/100g) (controls)
<i>P. aeruginosa</i>	63	55
<i>E. coli</i>	54	98
<i>S. aureus</i>	87	109
<i>B. subtilis</i>	96	66
<i>B. cinerea</i>	68	96

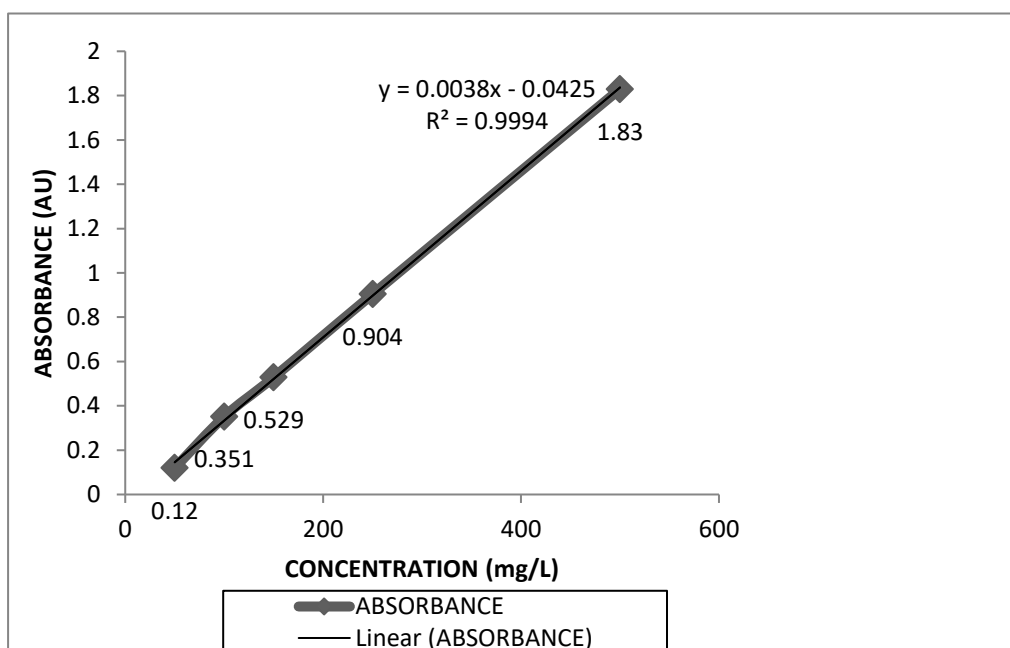


Figure 3.6. The calibration curve plotted against the absorbances of the gallic acid standards at 765nm.

The total phenolic content of the fruits incubated with the ZnO-starch NC paper was calculated to be between 54 and 96 mg/100g whilst for the controls, it was calculated to be between 55 and 109 mg/100g. Strawberries contain between 55 to 130 mg/100g of phenolic compounds meaning that even after incubation, the fruits still have total phenolic compounds which lie in the normal range for phenolic compounds (Aaby *et al.*, 2012). This shows that the total phenolic content quality assay was not useful in measuring the extent of spoilage of the strawberry fruits.

3.6.4 Vitamin C content

The Vitamin C content was calculated using the titration method. The Vitamin C content was calculated after incubation with ZnO-starch NC paper using Equation 8 and was found to be between 66 and 97 mg/100g for the fruits incubated with ZnO-starch NC paper whilst for fruits incubated without ZnO-starch NC paper, Vitamin C content was found to be between 61 and 92 mg/100g (Table 5).

Table 5. Vitamin C content of strawberries after incubation at 4°C with ZnO-starch NC paper and without the ZnO-starch NC paper (controls).

Pathogen used	Vitamin C (mg/100g) (NC paper)	Vitamin C (mg/100g) (controls)
<i>P. aeruginosa</i>	66	83
<i>E. coli</i>	90	69
<i>S. aureus</i>	81	87
<i>B. subtilis</i>	97	92
<i>B. cinerea</i>	89	61

Compounds such as ascorbic acids are used up increasing the pH of the fruit leading to undesirable sensory changes and spoilage. Therefore, after a few days of packaging, most fresh foods become spoiled and contain much illness-causing pathogens. The total Vitamin C content for the fruits incubated with ZnO-starch NC paper and for the controls was found to be 66 and 97 mg/100g and 61 and 92 mg/100g respectively. Freshly picked strawberries have a Vitamin C content of between 57 to 133 mg/100g which also shows that after the experiment, the fruits have a normal amount of Vitamin C content (Feliziani and Romanazzi, 2016). None of the fruits exhibited significantly different or lower quality parameters even among the test pathogens as there was not any significant change in these quality parameters during the experiment. This also shows that the Vitamin C content quality assay was not useful in measuring the extent of spoilage of the strawberry fruits.

3.6.5 Microbial count

The microbial count of the strawberries was calculated after the experiment using the spread plate method. The colony forming units per Gram (CFU/gram) (Equation 9) were found to be significantly higher in the fruits incubated without the ZnO-starch NC paper whilst the fruits incubated with the ZnO-starch NC paper showed up to 3-fold less CFU of bacteria (Table 6). The highest CFU/gram was found to be $>3 \times 10^4$ (*E. coli* and *P. aeruginosa*) whilst the lowest was found to be 2.5×10^3 CFU/gram (*B. subtilis*). For the fungal count, equation 10 was used to calculate CFU/g. Fruits inoculated with *B. cinerea* incubated with the ZnO-starch NC paper had a CFU/gram which is 3 times less than that of their controls.

Table 6. Microbial counts of the strawberries incubated at 4°C with the ZnO-starch NC paper and without the ZnO-starch NC paper (control).

Pathogen used	ZnO-starch NC paper (CFU/g)	Controls (CFU/g)
<i>B. subtilis</i>	2.5×10^3	8×10^3
<i>S. aureus</i>	3.3×10^3	9.5×10^3
<i>E. coli</i>	1.25×10^4	$>3.0 \times 10^4$
<i>P. aeruginosa</i>	1.86×10^4	$>3.0 \times 10^4$
<i>B. cinerea</i>	3.9×10^2	9.6×10^2

The microbial content of the fruits increased during storage. Due to the antibacterial and antifungal properties of the ZnO-starch NC paper, the test sample had about 3 times less microbial content compared to the controls. After 10 days the test samples were still physically acceptable whilst the controls had fungus and bacterial spoilage showing on their surfaces. It was expected that since the ZnO-starch NC paper was highly effective towards *B. subtilis* and *S. aureus*, the bacterial count will be the lowest on the fruits incubated with these pathogens. On the contrary, the results show that the bacterial count of the Gram-positive bacteria was the lowest of the 5 pathogens whilst the bacterial count of the Gram-negative bacteria was the highest, recorded as greater than 3×10^4 CFU/g since the plates were all too numerous to count. These results prove that *E. coli* and *P. aeruginosa* were resistant to the ZnO-starch NC. The health protection agency recommends that it's safe to consume ready to eat food with less than 10^5 CFU/g of bacteria and 10^3 CFU/g for fungi (Health protection agency, 2009). Both the test samples and controls had acceptable microbial content although the Gram-negative bacteria had CFU/g counts close to the unsafe level. The fungal count still showed that the ZnO-starch NC paper prevented the growth of mold on the fruits as the CFU/g was significantly lesser than that of the controls. The ZnO-starch NC paper was seen to have significant antibacterial and antifungal properties and can be used in the food industry for the preservation of fresh foods. Although the microbial counts were lesser for the test samples than the controls, the difference in microbial counts was seen to be statistically insignificant ($p > 0.05$).

Chapter 4: Conclusions and Recommendations for future work

4.1 Conclusion

ZnO and ZnO-starch NC were successfully synthesized using the hydrothermal method. Characterization of the nanomaterials showed that the ZnO had a hexagonal wurtzite structure and a crystalline size of between 30 – 35 nm. Addition of potato starch to the nanocomposite did not significantly affect the crystalline structure or the antibacterial and antifungal properties of the ZnO NPs. The antibacterial/antifungal assays showed that the ZnO-starch NC was significantly effective against *B. subtilis*, *S. aureus* and *B. cinerea* but not against *E. coli* and *P. aeruginosa* which was similar to reported literature on ZnO activity. Application of the ZnO-starch NC in packaging strawberries resulted in the reduction of weight loss and microbial growth compared to the controls. Total phenolic content, Vitamin C content and pH of both the test sample and controls were found to be in the normal range of recommended quality parameters of strawberries as there was not a significant change in intrinsic factors of the fruits during incubation. This was attributed to the number of microbes which was not sufficient to reduce the parameters to outside of their normal range. The appearance of the fruits showed the non-treated fruits as being spoiled after the experiment as they had visible soft rot and mold growth whilst the treated fruits were still acceptable without any visible microbial growth. The ZnO-starch NC paper improved the shelf life of the fruits during incubation at 4°C and resulted in fruits with acceptable quality and sensory qualities compared to the untreated fruits. As low temperatures help to slow the proliferation of microbes, the fruits incubated at a high temperature (27°C) were all spoiled by the end of the experiment. These results show that this novel nanomaterial could be utilized as an alternative packaging in prolonging the shelf life of strawberries as well as other fresh foods in the food industry.

4.2 Limitations

The strawberries were imported into Botswana and they would take a long time to be available for analysis which made analysis difficult and took longer than expected. At the time of doing the experiments, a farm that could provide fresh strawberries was not available in Botswana. The fruits were of different sizes which meant that they spoil at different rates and had different intrinsic factors hence the analysis was not always at the same conditions. Due to financial constraints and COVID restrictions, necessary equipment and reagents could not be purchased to perform certain tests that were vital for this project.

4.3 Recommendations for future work

The synthesized ZnO NPs and ZnO-starch NC were found to have desirable characteristics which could be useful in industry. To improve their properties and effectiveness, several factors need to be addressed. Hence, it is recommended that:

- a) Additional variations such as humidity, moisture content, gas levels and light spectrum should be investigated on their effect on the spoilage of the packaged fruits.
- b) The reusability and recyclability of the ZnO-starch NC should be investigated for potential commercial use.
- c) Further studies should be done to determine the effect of ZnO NPs on human cells as they are to be used for packaging consumables.
- d) The mode of action of the ZnO NPs can be studied further to understand the antibacterial and antifungal properties of the NPs to be able to maximize the antibacterial/antifungal application of the packaging.
- e) The resistant strain of bacteria to the ZnO-starch NC encountered in this study could be studied to understand their mode of resistance. This will enable to improvise on the ZnO size, concentration, shape or packaging so that there is an improved preservation capacity.
- f) The ZnO-starch NC paper should be used in packaging a wide range of fresh foods with different nutrient contents to determine the effectiveness of the packaging for use in the fresh food industry.
- g) The effectiveness of the packaging should be further studied, and the design should be improved.

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