ANTIMICROBIAL ACTIVITY OF SILVER NANOPARTICLES FOR THE CONTROL OF MICROBIAL SPOILAGE OF GREY OYSTER MUSHROOM (*PLEUROTUS PULMONARIUS*)

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FACULTY OF SCIENCE
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DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF BIOTECHNOLOGY

INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
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Field of Study: **BIOTECHNOLOGY**

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ANTIMICROBIAL ACTIVITY OF SILVER NANOPARTICLES FOR THE CONTROL OF MICROBIAL SPOILAGE OF GREY OYSTER MUSHROOM (*PLEUROTUS PULMONARIUS*)

ABSTRACT

Grey oyster mushroom (*Pleurotus pulmonarius)* is one of the most popular mushrooms in Malaysia and contains a high amount of nutrients. However, the fresh grey oyster mushroom is having short shelf life thus reducing its quality and economic value. Limited study on maintaining the fresh quality and prolonging the shelf life of the fresh grey oyster mushroom. Silver nanoparticles (Ag-NPs) are known to act as antimicrobial agents against a wider range of microorganisms. Therefore, this study aimed to identify the 11 spoilage bacteria strains isolated from rotten grey oyster mushrooms and evaluate the antimicrobial activity of different sizes of Ag-NPs (30.88 nm, 60.52 nm, 99.16 nm) to inhibit the growth of spoilage bacteria using the broth microdilution method. The spoilage bacterial strains were identified as *Raoultella ornithinolytica, Raoultella planticola, Klebsiella* sp., *Enterobacter cloacae,* and *Cedecea neteri* based on biochemical assay and 16S rRNA sequencing. At 50 µg/ml, Ag-NPs (30.88 nm) showed inhibition (ranged from 99.21-100%) on the growth of the 11 strains of spoilage bacteria. Also, Ag-NPs 30.88 nm and Ag-NPs 60.52 nm exhibited higher minimum inhibitory concentration to the spoilage bacteria as compared to Ag-NPs 99.16 nm. In conclusion, smaller-sized Ag-NPs have higher bacterial inhibitory capacity compared to large-sized Ag-NPs.

Keywords: silver nanoparticles, *Pleurotus pulmonarius,* pathogen, shelf life, mushroom spoilage bacteria.

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AKTIVITI ANTIMIKROBIAL SILVER NANOPARTIKEL UNTUK MENGAWAL KEROSAKAN MIKROBIAL TERHADAP CENDAWAN TIRAM KELABU *(PLEUROTUS PULMONARIUS)*

ABSTRAK

Cendawan tiram kelabu *(Pleurotus pulmonarius)* merupakan salah satu jenis cendawan yang popular di Malaysia dan cendawan ini kaya dengan sumber nutrisi. Walaubagaimanapun, kualiti cendawan tiram kelabu yang segar mempunyai jangka hayat yang rendah menyebabkan penurunan nilai ekonominya. Kajian untuk pengawalan kualiti dan untuk meningkatkan jangka hayat cendawan tiram kelabu adalah terhad. Silver nanoparticles (Ag-NPs) bertindak sebagai ejen antimikrobial terhadap mikroorganisma secara luas. Oleh itu, tujuan kajian ini ialah untuk mengenalpastikan 11 strain bakteria perosak yang diasingkan dari cendawan tiram kelabu yang layu dan untuk membuat penilaian aktiviti antimikrobial Ag-NPs dari saiz yang berbeza (30.88 nm, 60.52 nm, 99.16 nm) untuk menghalang penumbuhan bakteria perosak dengan menggunakan kaedah broth microdilution method. Strain bakteria perosak yang dikenalpastikan ialah *Raoultella ornithinolytica, Raoultella planticola, Klebsiella* sp*, Enterobacter cloacae* and *Cedecea neteri* berdasarkan kaedah biokimia dan penjujukan genom 16S rRNA. Ketika 50 µg/mL, Ag-NPs (30.88 nm) menunjukan penumbuhan dihalang (dari kadar 99.21-100%) untuk kesemua 11 strains bakteria perosak. Selain itu, Ag-NPs 30.88 nm dan 60.52 nm mempunyai nilai perencatan minimumyang tinggi terhadap bakteria perosak berbanding dengan Ag-NPs 99.16 nm. Kesimpulannya, Ag-NPs bersaiz kecil mempunyai keupayaan antibakterial yang tinggi berbanding dengan Ag-NPs yang bersaiz besar.

Kata kunci: nanopartikel perak, *Pleurotus pulmonarius,* bakteria perosak, jangka hayat, cendawan tiram kelabu.

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LIST OF SYMBOLS AND ABBREVIATIONS

$^{\circ}$C	:	degree Celsius
$\geq$	:	greater than or equal to
$>$	:	greater than
H_2O_2	:	hydrogen peroxide
$\leq$	:	less than or equal to
$<$	:	lesser than
µg/ml	:	microgram per milliliter
µl	:	microliter
%	:	percentage
BLAST	:	basic local alignment searching tool
Cu2+	:	copper ions
CFUg-1	:	colony forming unit per gram
CFU/ml	:	colony forming unit per milliliter
DNA	:	deoxyribonucleic acid
g	:	gram
KOH	:	potassium hydroxide
MIC	:	minimal inhibitory concentration
ml	:	milliliter
nm	:	nanometer
PCR	:	polymerase chain reaction
P. pulmonarius	:	*Pleurotus pulmonarius*
Rpm	:	revolutions per minute
rDNA	:	ribosomal DNA
RNA	:	ribonucleic acid
rRNA	:	ribosomal RNA
Ag-NPs	:	silver nanoparticles
Ag+	:	silver ion
Spp	:	species
TBE	:	tris-borate-EDTA buffer
UV	:	ultraviolet

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CHAPTER 1: INTRODUCTION

1.1 Introduction of study

Malaysia is one of the main producers of mushroom in the world and about 1000 tonnes of mushroom is being grown annually in Malaysia for local and export markets. In 2012, Malaysia was listed as one of the top five mushroom producers in the world (Amin et al., 2014). Many varieties of mushrooms are grown in Malaysia because of the favorable climate. The Oyster mushroom is the most common edible mushroom grown in Malaysia (Amin et al., 2014). Malaysia's Agrofood policy reported mushroom industry is estimated to contribute about RM300 million to the global national income and estimated the demand for mushrooms in local markets to be increased to 48,000 tonnes by the year 2020 (Amin et al., 2014).

The quality of the fresh mushroom is accessed by its appearances such as size, color, development stage, weight loss, maturity stage, and microbiological quality (Aguirre et al., 2008). However, fresh mushrooms are easily perishable and spoilt. High respiration rate and enzyme oxidation caused postharvest mushrooms to turn brownish in color and loss nutritional value. Also, high water content and high metabolic activity in mushrooms are the factors for spoilage and short shelf life (Mohebbi et al., 2012). Mushrooms with reduced quality lose commercial value because of water loss, microbial attack, senescence, and browning appearance. (Akbarirad et al., 2013; Jiang et al., 2012). The Gram-negative microorganisms (*Pseudomonas tolassi* and *P. fluorescens*) and yeast are the microorganisms that cause spoilage in mushrooms (Jiang et al., 2012).

The inhibition of water loss and reducing the uptake of oxygen are important factors to prolong the mushroom shelf life (Bira, 2015). The use of silver nanoparticles (Ag-NPs) is considered a safe and cost-effective method due to its antimicrobial properties (Sondi and Salopek, 2004). Previous studies reported that Ag-NPs could inhibit the growth of

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both Gram-positive and Gram-negative bacteria (Jiang et al., 2013a; Sondi and Salopek, 2004). Although there are concerns about Ag-NPs toxicity on human health, Le Quay and Stellacci (2015) reported that low doses of Ag-NPs possessed negligible systemic toxicity toward humans.

The antimicrobial mechanism of Ag-NPs involved penetrates the microbial cell and releases silver ions within the cells that interfere with the bacterial biological process such as respiration and cellular division eventually leading to cell death (Sondi and Salopek, 2004). However, the biological interaction and impacts of Ag-NPs on microbial cells are determined by the shape, surface area size, surface charge, coating, and dissolution rate (Wei et al., 2015).

1.2 Aim and objective of the study

The objectives of this study were to:

1) identify the isolated spoilage bacteria from the rotten grey oyster mushroom.
2) determine the antimicrobial capability of silver nanoparticles against the selected spoilage bacteria in grey oyster mushrooms.
3) compare the antimicrobial efficiency of silver nanoparticles of different sizes (30.88 nm,60.52 nm,99.16 nm).

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CHAPTER 2: LITERATURE REVIEW

2.1 Silver nanoparticles (Ag-NPs)

2.1.1 Characteristics of Silver nanoparticles (Ag-NPs)

Silver nanoparticles (Ag-NPs) presence in nature and a high abundance of Ag-NPs were found in an old silver mining area of Mexico, Texas riverine water, and estuarine water (Yu et al., 2013). Under specific environmental conditions, humic acids are capable of reducing Ag^+ and forming Ag-NPs (Akaighe et al., 2011). The production of Ag-NPs increases as the temperature increase. Studies have shown that under sunlight, the dissolved organic matter converts Ag^+ into Ag-NPs by the photochemical process in several hours (Yin et al., 2012). Some algae, bacteria, and plants can produce Ag-NPs as well. Alfalfa root tends to extract silver atoms and transform them into Ag-NPs (Yu et al., 2013).

The sizes and shapes of Ag-NPs are the crucial factors that determine the chemical, biological and physical properties of Ag-NPs (Zhang et al., 2016). Ag-NPs are less than 100 nm in size and the particles usually contain approximately about 20 – 15000 silver atoms (Shahverdi et al., 2007). Various applications of Ag-NPs including antibacterial agents, in the industrial, household, and healthcare-related products, consumer products, medical device coatings, optical sensors, and cosmetics, in the pharmaceutical industry, the food industry, in diagnostics, orthopedics, drug delivery, as anticancer agents, and as tumor-killing anticancer drugs can be seen nowadays (Zhang et al., 2016).

2.1.2 Antimicrobial Properties of Silver nanoparticles (Ag-NPs)

Ag-NPs are an alternative antimicrobial agent to the current antibiotics and possess the ability to overcome the problem of microbial resistance. This unique property of Ag-NPs is enhanced by the large surface-to-volume ratios and crystallographic surface structure (spherical, triangular nanoplates, and rod) (Zhang et al., 2016). Ag-NPs

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inhibited the growth of Gram-positive (*Clostridium, Streptococcus, Staphylococcus, Bacillus, Listeria,* and *Enterococcus)* and Gram-negative bacteria (*Acinetobacteria, Pseudomonas, Escherichia, Vibrio* and *Salmonella*) excluding antibiotic-resistant bacteria (Vanomycin-resistant bacteria) (Shahverdi et al., 2007; Zhang et al, 2016). Shahverdi et al. (2007) reported that Ag-NPs coupled with various antibiotics such as amoxicillin, clindamycin, penicillin G, vancomycin, and erythromycin increase the antimicrobial properties against *E. coli* and *Staphylococcus.* The triangular nanoplates structure imposes the highest antimicrobial property compared with other shapes of Ag-NPs (Hwang et al., 2008).

The mechanisms of the antimicrobial effect of Ag-NPs are still immensely studied. The mode of antimicrobial action of Ag-NPs is penetrating the cell wall of the Gram-negative bacteria and causing bacteria to increase the permeability of the cell. Ag-NPs penetration causes the formation of free radicals which increases cell damage and eventually kills the bacteria. Studies conducted by Hwang et. Al (2008), have shown that silver ions (Ag^+) released by Ag-NPs into the bacteria cause reactive oxygen species, the synergetic mechanism of both Ag-NPs and Ag^+ causes the bacteria to die. Ag^+ interacts with the enzymes of bacteria and interferes with the phosphorus-containing bases in the cell. DNA is damaged when the silver ions interact with DNA in the cell which inhibits the process of cell replication (Hwang et al., 2008). Ag-NPs are potential to be used as the antifungal agent against genera of *Aspergillus*, *Saccharomyces,* and *Candida* (Wright et al., 1999).

Silver nanoparticles have a better bactericidal effect compared to copper nanoparticles as silver ion binds to cysteine residue without a homeostasis mechanism and silver ion makes cysteine lose its function as functional amino acid while Cu^{2+} still undergoes the homeostasis process upon binding to the cysteine group. Other than that, Ag^+ is a non-

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essential element to the bacteria, unlike Cu^{2+} which is an essential element, and Ag^{+} forms irreversible binding with the cysteines. The binding of Ag^{+} on cysteine in the bacteria becomes toxic for the enzymatic mechanism of the bacteria and disrupts the respiratory electron transport chains (Slavin et al., 2017).

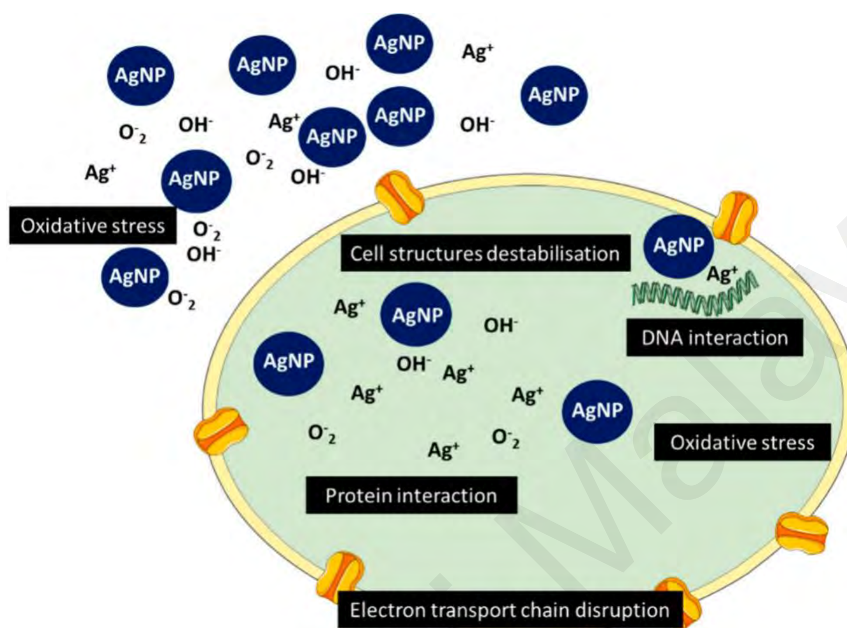

Figure 2.1: **Schematic representation of antibacterial properties of Ag-NPs (Source: Sánchez-López et al., 2020)**

Figure 2.1 shows the schematic representation of Ag-NPs mode of killing the Gram-negative bacteria. Ag-NPs have four different mechanisms for killing Gram-negative bacteria such as being attracted to the negatively charged bacterial surface, reducing the stability of the cell wall of bacteria, production of ROS organisms, and modulating the signal transduction pathways (Sánchez-López et al., 2020).

2.1.3 Silver nanoparticles (Ag-NPs) in food packaging

The use of packaging is to contain, handle, present, protect and distribute the products and goods. Without altering the properties of the product, antimicrobial materials can be

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added to plastics before the polymerization process. Ag-NPs addition in packaging materials has many benefits such as increased impermeability, nanoparticles packaging blocks the ultraviolet light and providing an antimicrobial surface (Song et al., 2011). Ag-NPs are used in the food technology industry as food packaging and food storage material (Carbone et al., 2016). There are two types of Ag-NPs food packaging methods:

a) Improved packaging where Ag-NPs are mixed with the synthetic polymer or with the matrix.

b) Active packaging where the Ag-NPs are used directly on the product.

Ag-NPs can withstand high temperature and has low volatility, thus has been chosen as an effective antimicrobial agent for packaging (Carbone et al., 2016). Ag-NPs can be used in both biodegradable films and on polymers against spoilage bacteria. The addition of nanoparticles can protect the content of food from the oxidizing effects of UV irradiation. This is due to the difference in the wavelength of light absorbed by the nanoparticles which create UV protection (Duncan et al., 2011).

2.2 Oyster mushroom

Oyster mushroom belongs to the genus *Pleurotus* and is one of the most cultivated mushrooms in the world (Adebayo and Oloke, 2003). This mushroom grows on lignocellulolytic materials such as sawdust and agricultural wastes. Mushrooms are rich in protein and contain a good source of nutrients (Adebayo and Oloke, 2003). *Pleurotus* species mushrooms had been used traditionally for treating many ailments and contain medicinal properties such as anti-tumor, anti-inflammatory, anti-viral, and immune-stimulatory (Patel et al., 2012). *Pleurotus* mushroom contains many complex compounds such as peptides, proteins, lectins, starch, lipopolysaccharides, triterpenoids, and lipids (Patel et al., 2012; Adebayo and Oloke, 2017; Chang and Buswell, 2003).

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2.3 Factors affecting mushroom spoilage

Mushrooms are perishable like green vegetables where the cell content and membrane integrity are easily lost and caused deterioration of the cell structure. Deterioration of cells causes loss of aroma and flavor of the mushroom (Singh et al., 2010). Rapid water loss in mushrooms affects the nutritional value of the mushroom and causes microbial invasion of the mushroom (Singh et al., 2010).

In the horticulture product industry, color is one of the important aspects for consumers. Browning in mushrooms has a huge impact on economic value (Singh et al., 2010). The browning effect in mushrooms is mainly due to aging and postharvest effect. The factors that cause mushroom browning include the phenolic oxidation process, the concentration of active polyphenol oxidase, water activity, oxygen amount in tissue, pH, and temperature (Singh et al., 2010).

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2.4 Spoilage bacteria in mushroom

High bacteria population presence in normal healthy *Agaricus* mushroom with the total bacteria concentration range from 6.3 to 7.2 log CFU g^{-1} (Singh et al., 2010). The mushroom spoilage occurs due to microbial activities during the storage (Singh et al., 2010). Usually, the water activity of more than 0.98 and a neutral pH value give an ideal condition for microbial growth (Singh et al., 2010). Singh et al. (2010) found that about 54% of spoilage bacteria in *Agaricus* mushroom comprises fluorescent pseudomonads species (genus *Pseudomonas*) followed by flavobacteria population (10%). Yeasts and molds are considered highly populated which is ranged 3 log CFU g^{-1} and 6 log CFU g^{-1} in fresh mushrooms. *Verticilium maltousei* and *Pseudomonas tolassi* were reported for causing blotch formation in the fresh *Agaricus* mushroom (Singh et al., 2010). The signs of blotching disease include dark, sunken, and brown spots on the mushrooms (Singh et

al., 2010). *Pseudomonas tolassi* contamination and exposure to the tolassin toxin may activate the tyrosinase activity in the mushroom. *Pseudomonas gingeri* is reported to cause a severe yellowish lesion on a mushroom while *Pseudomonas reactans* causes mild infection on mushroom by forming superficial discoloration (Singh et al., 2010).

Singh et al. (2010) reported that the bacterial load of *Agaricus* mushroom stored at 4°C for 24 hours increased from 7.3 to 8.4 log CFU g^{-1}, while yeast population increased from 6.9 log CFU g^{-1} to 8.0 log CFU g^{-1}. Singh et al. (2010) has also shown that mesophilic bacteria could increase during *Agaricus* mushroom storage. Spoilage bacteria that are isolated in post harvested fresh *Pleurotus* mushrooms are *Bacillus cereus, Staphylococcus aureus,* and *Escherichia coli* (Singh et al., 2010).

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CHAPTER 3: MATERIALS AND METHODS

3.1 Study design

This study consists of three parts. In part I, bioassay and biochemical test of spoilage bacteria from rotten *Pleurotus pulmonarius* using oxidase test, catalase test, motility test, and Gram staining. In part II, the species identification of mushroom spoilage bacteria using 16S rRNA gene polymerase chain reaction. In part III, the antimicrobial property of Ag-NPs was tested against spoilage bacteria using the broth micro-dilution method. The flow of this research study is outlined in Figure 3.1.

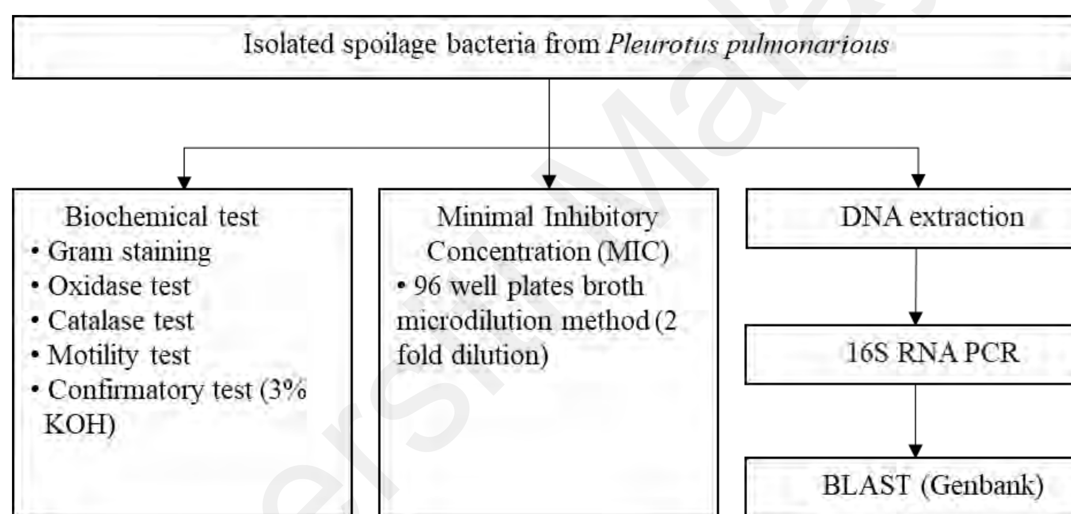

Figure 3.1: The flow of research study

3.2 Materials

A total of eleven spoilage bacteria were isolated from rotten *Pleurotus pulmonarius* mushrooms. Three different sizes of Ag-NPs (30.88 nm, 60.52 nm, and 99.16 nm) samples with the concentration of 71000 µg/ml in Demethylacetamide (DMAC) as the stock solution was obtained from Nanotechnology and Catalysis Research Center, Universiti Malaya. Sulfamethoxazole (SMX) was prepared as the positive control (1000 µg/ml) and sterile Mueller Hinton broth was used as the negative control.

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3.3 Bacterial culture

A total of eleven spoilage bacteria were isolated from rotten *Pleurotus pulmonarius* mushrooms in the previous study were included in this study. The bacteria strains were revived from the glycerol stocks by transferring 100 Ul of glycerol culture into 10 ml of nutrient broth and incubated at 35°C for 18-24 hours. The enriched broth was then streaked onto nutrient agar that was then incubated at 35°C for 18-24 hours before the isolate was then maintained on Nutrient agar slant as working cultures.

3.4 Gram staining

The specimen of bacteria was heat-fixed on the microscope slide for 1 minute with a crystal violet staining reagent. The slide was gently washed with tap water for 2 seconds. The slide was covered with Gram's iodine and waited for 1 minute and it was washed gently with tap water for 2 seconds. The slide was flooded with the decolorizing agent and waited for 15 seconds. Followed by covering with counterstain known as safranin and waited for 1 minute. The slide was washed with tap water and blot dried with absorbent paper. The result was observed and examined under a 100X objective lens using the light microscope. The Gram reaction on the bacteria was described.

3.5 Biochemical test

3.5.1 Oxidase test

The filter paper method was used to test the oxidase reaction of the eleven bacteria strains. The filter paper was soaked with the substrate 1% tetramethyl-p-phenylenediamine dihydrochloride solution (Kovac's oxidase reagent) and the freshly grown bacteria culture on Nutrient agar was scraped using a sterile toothpick and smeared on the filter paper. The color change during the reactions was observed in 10 seconds and recorded, and deep purple to the blue color formation during the process was recorded as a positive reaction. The test was done in duplicate.

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3.5.2 Catalase test

The microscope slide was placed inside a Petri dish. By using a sterile wooden toothpick, a small amount of 24-hour old bacteria culture on a nutrient agar plate was placed onto the microscope slide. By using a dropper, a drop of 3% H_2O_2 was dropped onto the slide. Reactions were observed and recorded. Strains that formed bubbles were labeled as a positive reaction. The test was done in duplicate.

3.5.3 Motility test

Motility test was used for the eleven strains of mushroom spoilage bacteria using Triphenyl Tetrazolium Chloride (TTC) motility agar. The bacteria strain was inoculated into tubes of TTC motility agar by stabbing with a needle to reach the bottom of the tube. The agar was incubated at 30°C and the spread of the inoculum away from the inoculation line was observed for each strain of bacteria. An intact straight stab line was recorded as a non-motile strain. While the spoilage bacteria stains stab line that diffused out into the medium was recorded as motile strain. The test was done in duplicate.

3.5.4 Potassium Hydroxide test

Potassium hydroxide was used as the confirmatory test for the bacteria Gram staining. A drop of potassium hydroxide (3 %) was mixed with a loop of bacteria. The formation of viscous string was confirmed as Gram-negative bacteria, while Gram-positive bacteria do not form viscosity. The test was carried out in duplicate.

3.6 Antibacterial Assay

3.6.1 Preparation of bacteria colony suspension

For each bacteria isolate, three to five morphologically similar colonies from the fresh nutrient agar plate were picked using a sterile loop. Then the growth was transferred into

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a sterile capped glass tube containing 0.9 % saline. The solution was mixed using a vortex mixer. The turbidity of all the eleven bacteria suspensions was adjusted to 0.5 McFarland (1 X 10^8 CFU ml^{-1}) and sulfamethoxazole was used as the positive control.

3.6.2 Minimal inhibitory concentration (MIC)

Three different sizes of Ag-NPs (30.88 nm, 60.52 nm, and 99.16 nm) samples with a concentration of 71000 µg/ml were used as stock solution. The samples were then diluted with 5 ml of Mueller hinton broth to obtain a concentration of 100 µg/ml. Tests were carried out in duplicate for each Ag-NPs sample using 96-well plates based on the method described by Wiegand et al. (2008).

The sample was further diluted with sterile saline (0.85%) to 50 µg/ml, 25 µg/ml, 12.5 µg/ml, 6.25 µg/ml, 3.13 µg/ml and 1.56 µg/ml. Demethylacetamide (DMAC), Sulfamethoxazole (SMX) were used as the positive control (1000 µg/ml) and sterile Mueller Hinton broth was used as the negative control. Bacteria sample was picked from the colony.

The plate was incubated at 37°C for 24 hours. After 24 hours of incubation, the turbidity of the wells was compared with the sterile control wells. The growth endpoint was determined. The lowest concentration that inhibits the growth of bacteria is considered as MIC. After the observation, the 96-well plates were incubated at 37°C for 30 minutes and the 96-well plates were shaken, the optical density of cell suspension was read using 600 nm wavelength using a microplate reader.

3.7 DNA extraction method

A total of 100 µl sterile water was added into a 1.5 ml microcentrifuge tube. One colony of bacteria was picked and mixed in sterile water. The solution was incubated at

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100 °C for 5 minutes and then the solution was incubated at -20°C for 5 minutes. The solution was centrifuged at 1000 rpm for 1 minute. The supernatant was aliquot and transferred into a new 1.5 ml microcentrifuge tube and stored at -20 °C.

3.8 Preparation of agarose gel

A total of 0.2 g of agarose powder was dissolved in 20 ml of TBE buffer. The mixture was heated in a microwave for 5 minutes. After that, the mixture was cooled down, and 1 µl of red stain dye was added and mixed. The agarose mixture was poured on the gel block and it solidified. TBE buffer was poured once the gel has solidified.

3.9 Polymerase chain reaction

PCR reaction was carried out in a final volume of 25 µl of PCR master mix which. Contained GoTaq (5X), $MgCl_2$ (25 mM), dNTP (10 mM), Taq (1 U/ml), deionized water, DNA, 27F primer (10 mM) and 1492 R primer (10 mM), For negative control, PCR mastermix and deionized water was added instead of DNA template. PCR reaction was carried out in a thermocycler programmed with an initial denaturation step at 95°C for 5 minutes, denaturation step at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and elongation at 72°C for 30 seconds, followed by a final elongation step at 72°C for 7 minutes.

After completing the PCR reaction, 3 µl of PCR product was mixed with 0.5 µl of loading dye and ran agarose gel electrophoresis. The electrophoresis process was run for 40 minutes at 100 Volt. The 2500bp ladder was used as control. The gel was observed using the UV transilluminator. All the samples were outsourced to the DNA sequencing lab for sequencing.

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3.10 Basic local alignment search tool (BLAST)

After PCR, 3 µl of PCR solution was mixed with 0.5 µl of loading dye and ran agarose gel electrophoresis process for 40 minutes at 100 Volt. The DNA samples were outsourced to DNA sequencing Lab for sequencing.

Table 3.1: The reagents used for PCR amplification

REAGENT	STOCK CONCENTRATION	VOLUME (µl)	FINAL CONCENTRATION
Go Taq	5X	5	1 X
$MgCl_2$	25 mM	1.5	1.5 mM
Dntp	10 mM	0.5	0.2 mM
Taq (5U/ml) = (1U/ml)	1 U/ µl	0.5	0.5 U/µl
Deionized water		15.5	
DNA		1	
27F primer	10 mM	0.5	0.2 mM
1492R primer	10 mM	0.5	0.2 mM
TOTAL		25.0	

3.11 Data analysis

Minimal inhibitory concentration is determined after the incubation of 96 well plates (broth microdilution method), the lowest concentration that inhibited visible growth of the mushroom spoilage bacteria is determined as the MIC value. All statistical analysis was performed using SPSS. Multiple comparison tests were conducted with Two-tailed ANOVA, Tukey HSD, Duncan, and LSD for Ag-NPs against mushroom spoilage bacteria.

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CHAPTER 4: RESULTS

4.1 Identification of spoilage bacteria from rotten *Pleurotus pulmonarius*

PCR amplification successfully amplified the 16s rDNA gene from only 10 out of the 12 bacteria identified obtained from the rotten *Pleurotus pulmonarius* (Figure 4.1). PCR of the bacterial isolate RedV failed to yield any amplification despite multiple trials that include re-extraction of crude genomic DNA from the isolate (Figure 4.1). Failure to yield a positive amplification as observed in this case could be due to: (i) too much DNA and it inhibits the PCR reaction; (ii) too little or no DNA that it is not enough for PCR amplification; and (iii) the presence of some internal inhibitor such as bacterial carbohydrates that blocks the PCR reaction (Canene-Adams, 2013), the acidic polysaccharides of the bacteria might have inhibited PCR amplification (Monteiro et al., 1997). The cross-linkage between protein and DNA via carbohydrate can cause inhibition of PCR (Reiss & Rutz, 1999). The inhibition of carbohydrates is termed incidental contamination (Soliman et al., 2017). Other than that, the DNA concentration of the 32C sample might be too numerous or too little as the PCR was a failure (Reiss & Rutz, 1999).

However, due to time constraints, all these three possibilities that may cause the failure in yielding a positive amplification for RedV and 32C isolate was not checked out. Therefore, the identity of RedV and 32C isolate remained undetermined in this study.

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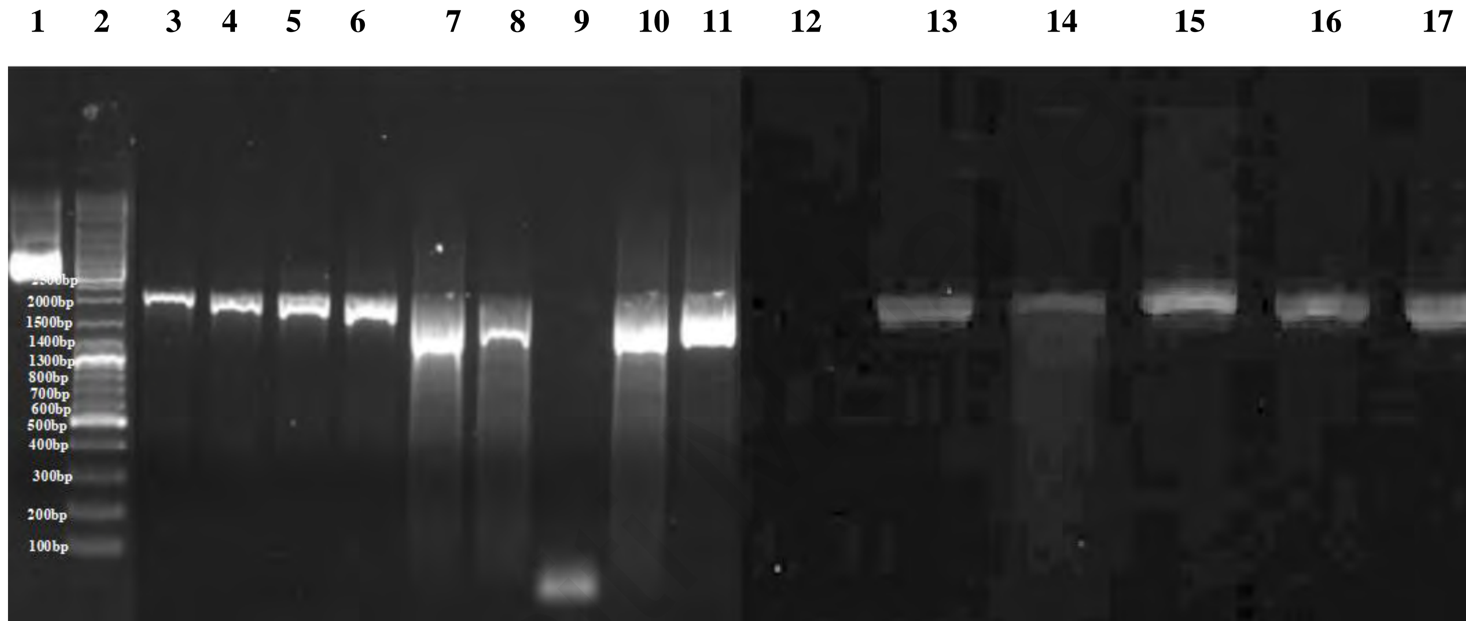

Figure 4.1: PCR profile of isolated spoilage bacteria from *Pleurotus pulmonarius* by 1% agarose gel electrophoresis. Lane 1 : positive control ; Lane 2 : Runsafe ladder 100 bp; Lane 3 : 37V; Lane 4 : 29; Lane 5 : 37C; Lane 6 : 31C; Lane 7 : 32C; Lane 8 : 17C; Lane 9 : RedV; Lane 10 : 31; Lane 11 : 35; Lane 12 : negative control; Lane 13 : RedV; Lane 14 : RedV ; Lane 15 : 36C; Lane 16: 28; Lane 17: 28.

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Based on the 16s rDNA gene sequencing and biochemical assays, bacteria strain 28 was identified as *Cedecea neteri* a Gram-negative, oxidase-negative, catalase-positive, motile, and rod-shaped bacteria. NCBI BLASTn demonstrated that the strain had 99% sequence identity with 16S rDNA of *C. neteri* strains (GenBank accession nos. CP009459, CP009458, KP322605, and CP009451).

Bacteria strain 37C was identified as *Klebsiella* sp a Gram-negative, oxidase-negative, catalase-positive, non-motile, and rod-shaped bacteria. NCBI BLASTn demonstrated that the strain had 99% sequence identity with 16S rDNA of *Klebsiella* sp strains (GenBank accession nos. EU888474 and LR134230).

The bacteria strain 31C was identified as *Raoultella ornithinolytica* a Gram-negative, oxidase-negative, catalase-positive, non-motile, and rod-shaped bacteria. NCBI BLASTn demonstrated that the strain had 97% sequence identity with 16S rDNA of *Raoultella ornithinolytica* strains (Genbank accession nos. CP017802 and CP013338).

The bacteria strain 29 was identified as *Raoultella ornithinolytica,* a Gram-negative, oxidase-negative, catalase-positive, non-motile, and rod-shaped bacteria. NCBI BLASTn demonstrated that the strain had 97% sequence identity with 16S rDNA of *Raoultella ornithinolytica* strains (Genbank accession nos. CP017802, CP013338, CP038281, and CP023888).

The bacteria strain 37V was identified as *Enterobacter cloacae*, Gram-negative, non-motile, catalase-positive, oxidase-negative, and rod-shaped. NCBI BLASTn demonstrated that the strain had 97% sequence identity with 16S rDNA of *Enterobacter cloacae* strains (Genbank accession nos. KY930709, MH399227, MH021673, and CP040827).

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The bacteria strain 35, 31, 36C, and 17C were identified as *Raoultella planticola.* These bacteria were all found to be rod-shaped Gram-negative, oxidase-negative, catalase-negative, and non-motile bacteria. NCBI BLASTn demonstrated that the strain had ≥97% sequence identity with 16S rDNA of *Raoultella planticola* strains (Table 4.1).

The strain RedV was an unidentified strain, the strain was Gram-negative, oxidase-negative, catalase-positive, non-motile, and cocci-shaped bacteria. The strain 32C was an unidentified strain, the strain was Gram-negative, oxidase-negative, catalase-positive, non-motile, and rod-shaped bacteria. RedV sample was done twice as in the first trial it did not has bands and in the second test RedV has band in the PCR but no significant similarity was found in the BLAST might be due to technical errors. 32C has band and no significant similarity was found in the BLAST. Table 4.1 summarized the characteristics and blast results and identity of each of the bacteria isolates included in this study.

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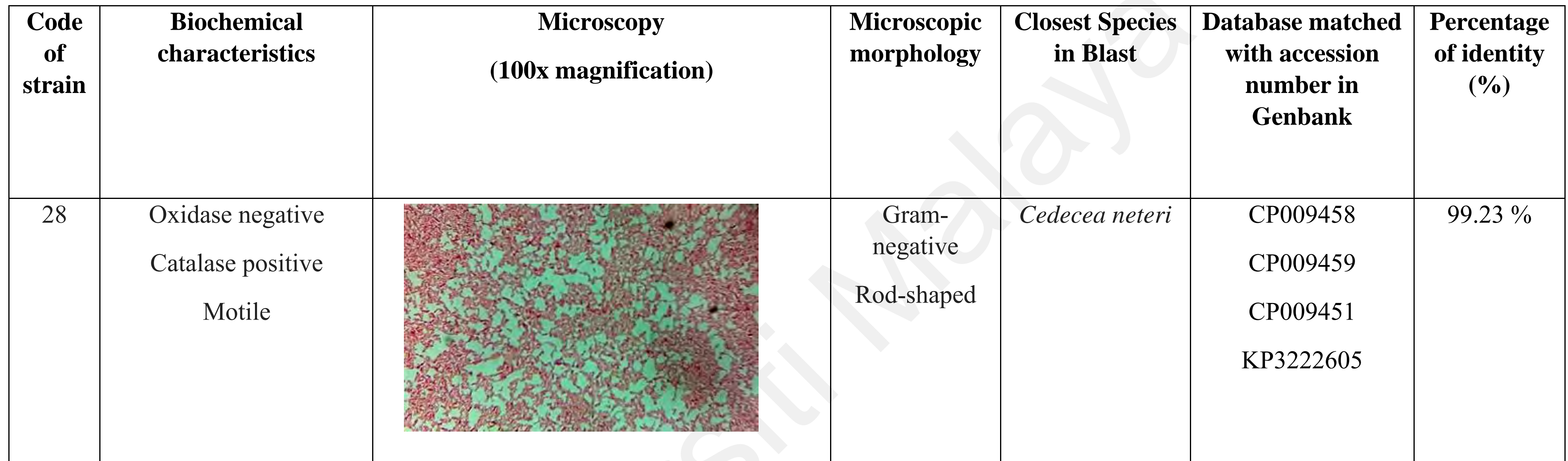
Table 4.1: Characteristics, blast results, and identity of bacteria isolates.

Code of strain	Biochemical characteristics	Microscopy (100x magnification)	Microscopic morphology	Closest Species in Blast	Database matched with accession number in Genbank	Percentage of identity (%)
28	Oxidase negative Catalase positive Motile		Gram-negative Rod-shaped	*Cedecea neteri*	CP009458 CP009459 CP009451 KP3222605	99.23 %

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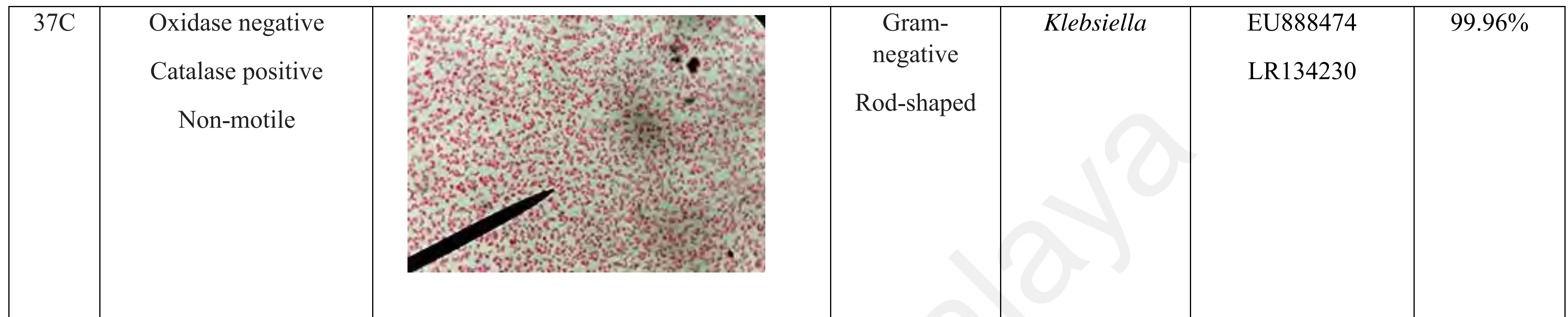
37C	Oxidase negative Catalase positive Non-motile		Gram-negative Rod-shaped	*Klebsiella*	EU888474 LR134230	99.96%

Table 4.1, continued.

31C	Oxidase negative Catalase positive Non-motile		Gram-negative Rod-shaped	*Raoultella Ornithinolytica*	CP017802 CP013338	97.45%

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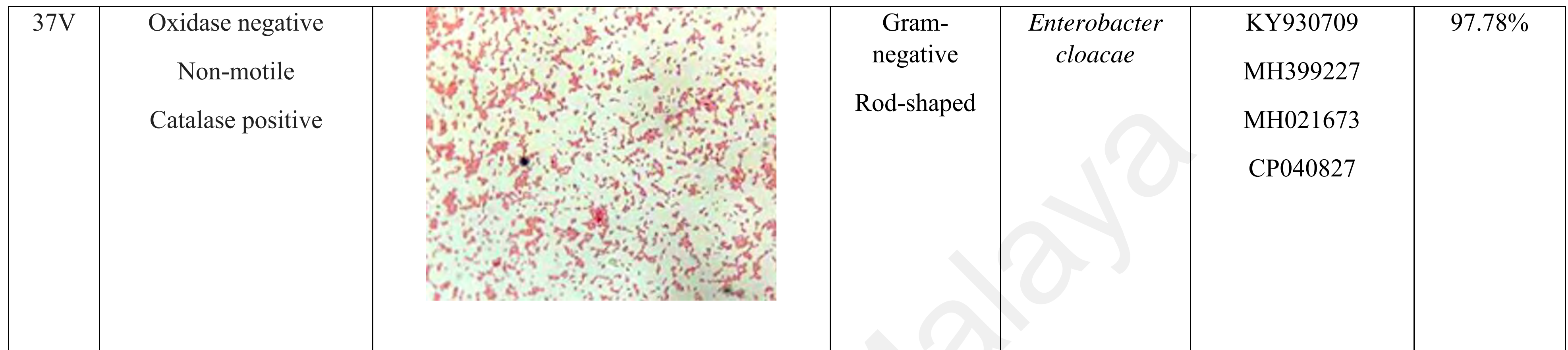
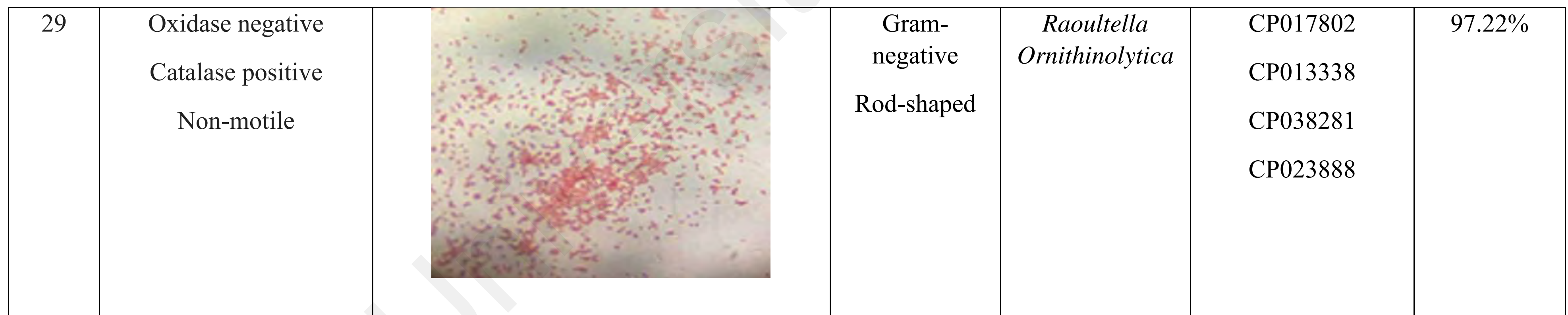
37V	Oxidase negative Non-motile Catalase positive		Gram-negative Rod-shaped	*Enterobacter cloacae*	KY930709 MH399227 MH021673 CP040827	97.78%

Table 4.1, continued.

29	Oxidase negative Catalase positive Non-motile		Gram-negative Rod-shaped	*Raoultella Ornithinolytica*	CP017802 CP013338 CP038281 CP023888	97.22%

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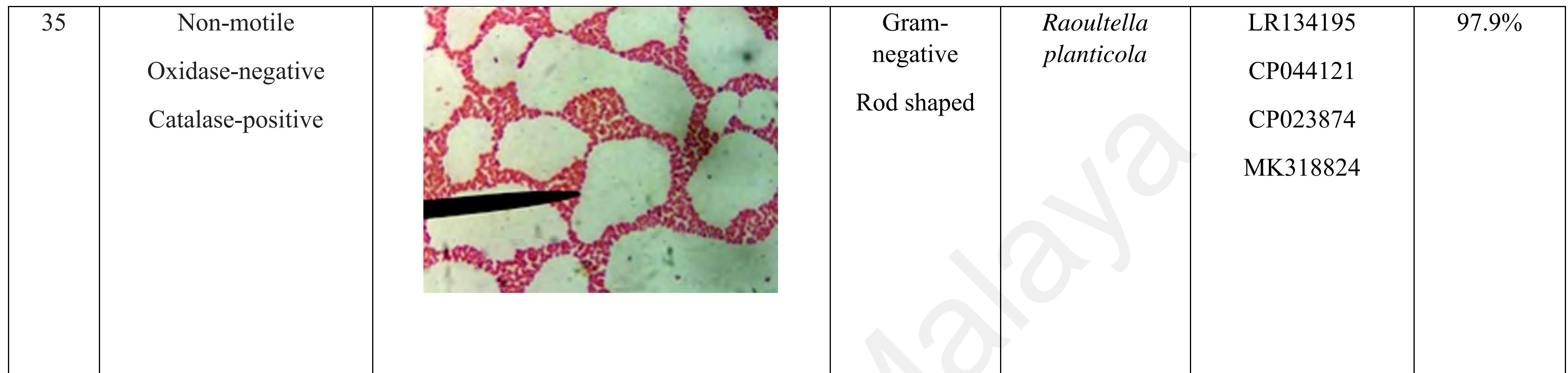
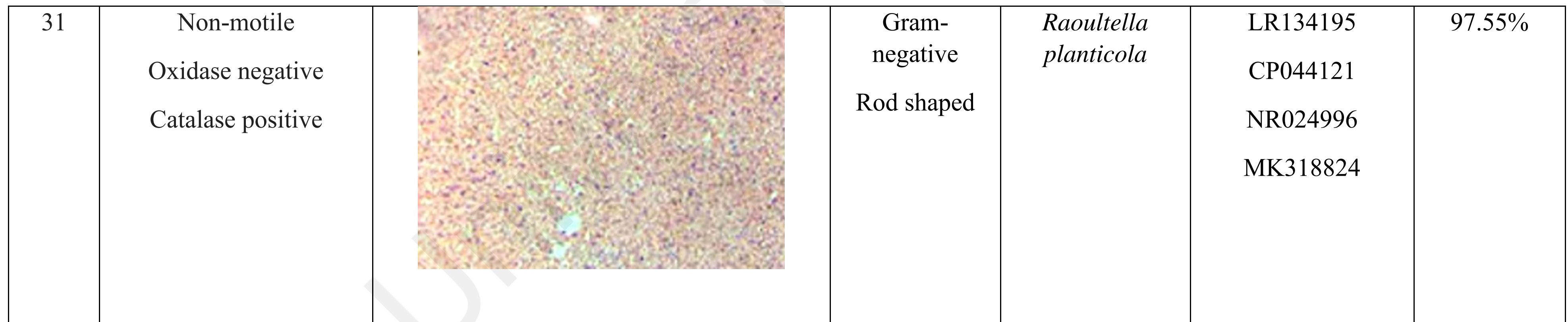


35	Non-motile Oxidase-negative Catalase-positive		Gram-negative Rod shaped	*Raoultella planticola*	LR134195 CP044121 CP023874 MK318824	97.9%

Table 4.1, continued.



31	Non-motile Oxidase negative Catalase positive		Gram-negative Rod shaped	*Raoultella planticola*	LR134195 CP044121 NR024996 MK318824	97.55%

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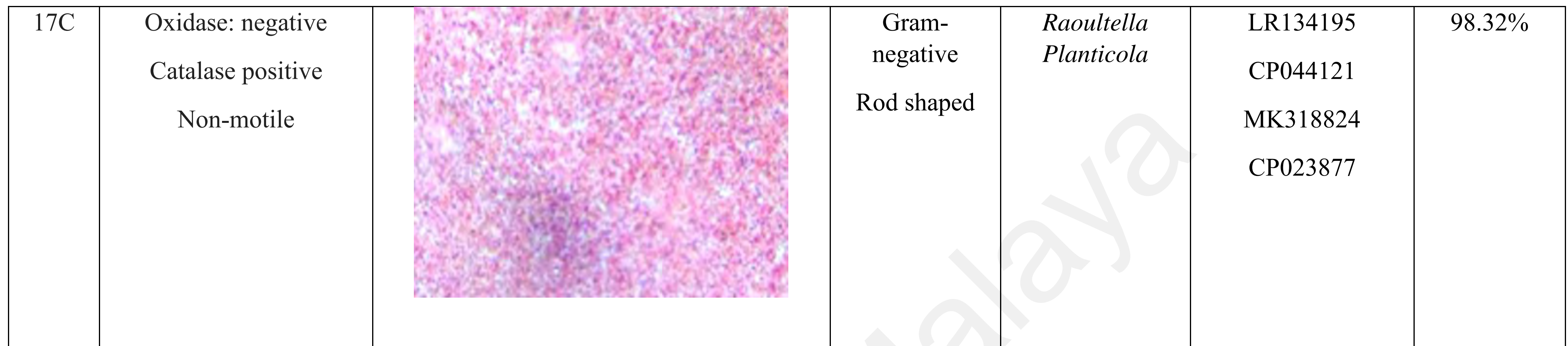
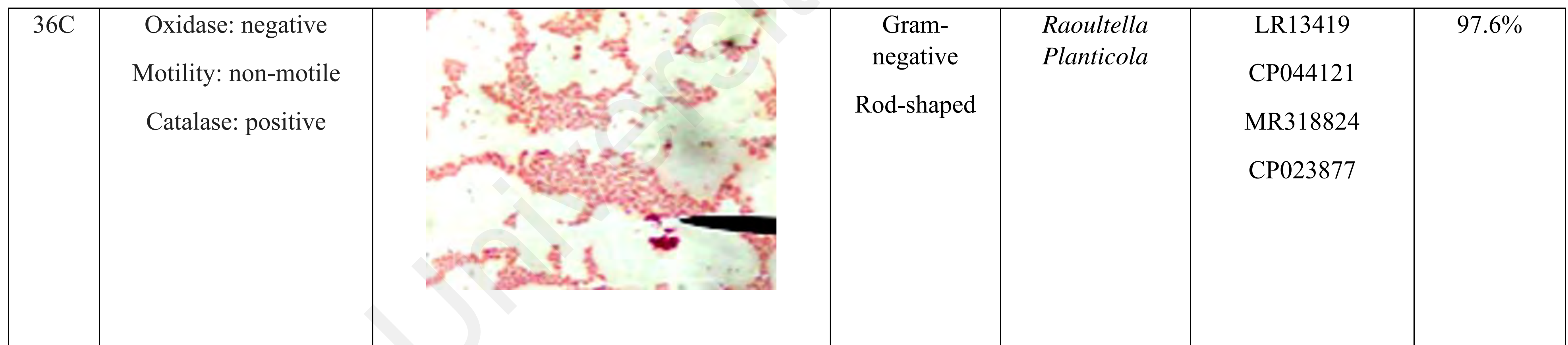
17C	Oxidase: negative Catalase positive Non-motile		Gram-negative Rod shaped	*Raoultella Planticola*	LR134195 CP044121 MK318824 CP023877	98.32%

Table 4.1, continued.

36C	Oxidase: negative Motility: non-motile Catalase: positive		Gram-negative Rod-shaped	*Raoultella Planticola*	LR13419 CP044121 MR318824 CP023877	97.6%

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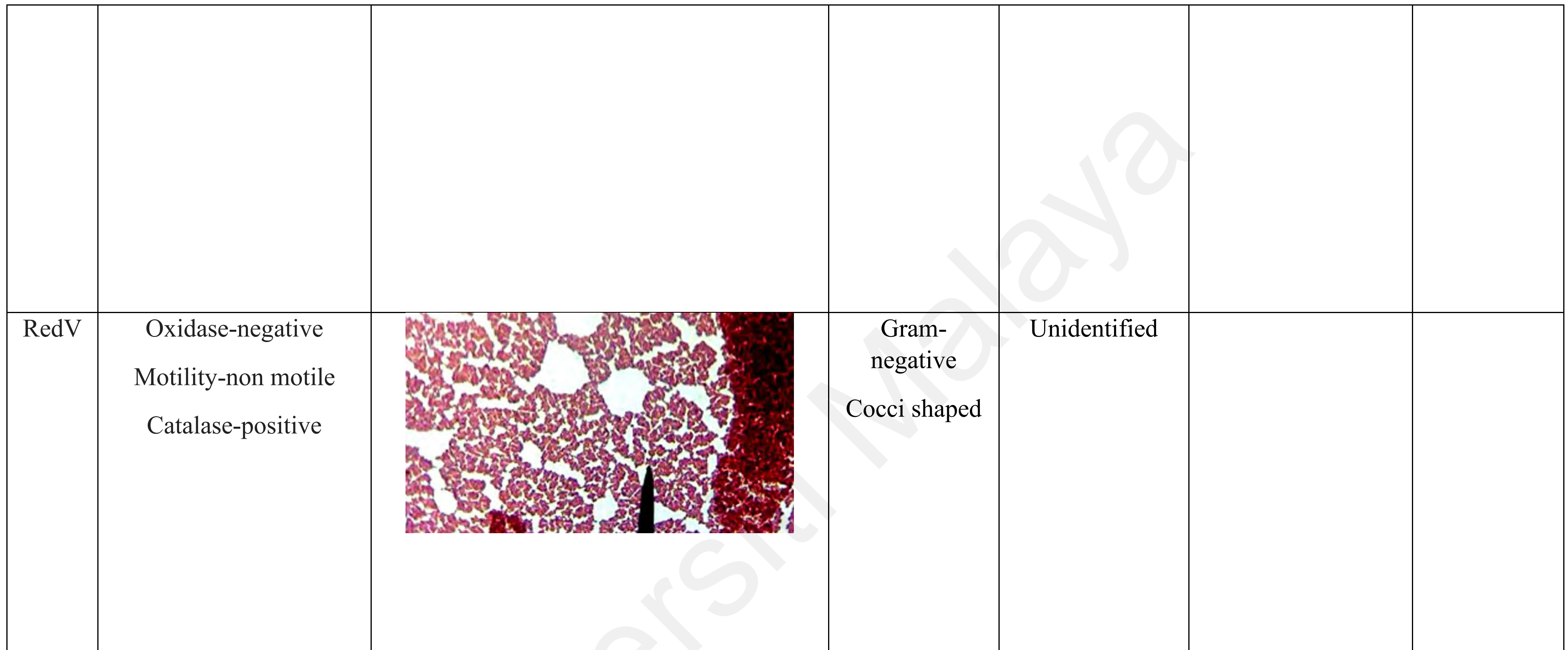
RedV	Oxidase-negative Motility-non motile Catalase-positive		Gram-negative Cocci shaped	Unidentified		

Table 4.1, continued.

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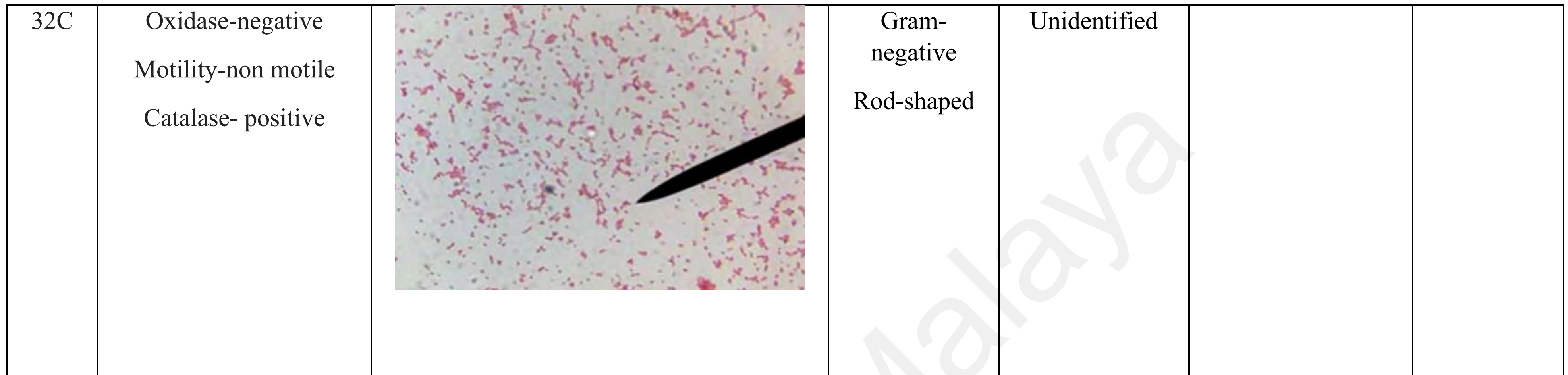
32C	Oxidase-negative Motility-non motile Catalase- positive		Gram-negative Rod-shaped	Unidentified			

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4.2 Determination of the antimicrobial capacity of the silver nanoparticles

4.2.1 Growth inhibition of the mushroom spoilage bacteria by the silver nanoparticles

Ag-NPs with different sizes showed variable differences in the antimicrobial capacity ($F=13.342$, $p<0.00001$). This experiment was conducted with the highest concentration of Ag-NPs (50 µg/ml) for each diameter of Ag-NPs. The result showed that Ag-NPs with the smallest diameter (30.88 nm) had the highest capacity in inhibiting the growth of the spoilage bacteria which was (99.89±0.21%); followed by Ag-NPs 60.52 nm (98.84±1.53%), and the least effective was Ag-NPs 99.16 nm (32.73±30.75%). Only the highest inhibitory capacity for each spoilage bacteria was evaluated. A total of 50 µg/ml Ag-NPs (30.88 nm) inhibited the growth of all the 11 strains of spoilage bacteria tested ranging from 99.21-100%, with the lowest growth inhibition observed for *Raoultella planticola* strain 31 (Figure 4.2). However, the inhibitory data revealed no significant difference between the values of growth inhibition capacity for each spoilage bacteria. Interestingly, the variation in the bacterial growth inhibition across the spoilage bacteria tested increased with the increase in the size of the Ag-NPs. The range of growth inhibition of Ag-NPs (60.52nm) was 94.97-100%, with the lowest inhibition recorded for *Raoultella planticola* strain 36C; while the bacterial growth inhibition of Ag-NPs (99.16nm) ranged from 0% to 78.24% (Figure 4.2). It is noteworthy to point out that the consistency in bacteria inhibition was low for Ag-NPs 99.16 nm, in which the result for biological replicates differed a lot. Both negative controls of Mueller-Hinton broth (without bacteria culture) and Dimethylacetamide (without culture) remain clear after 24 hours of incubation.

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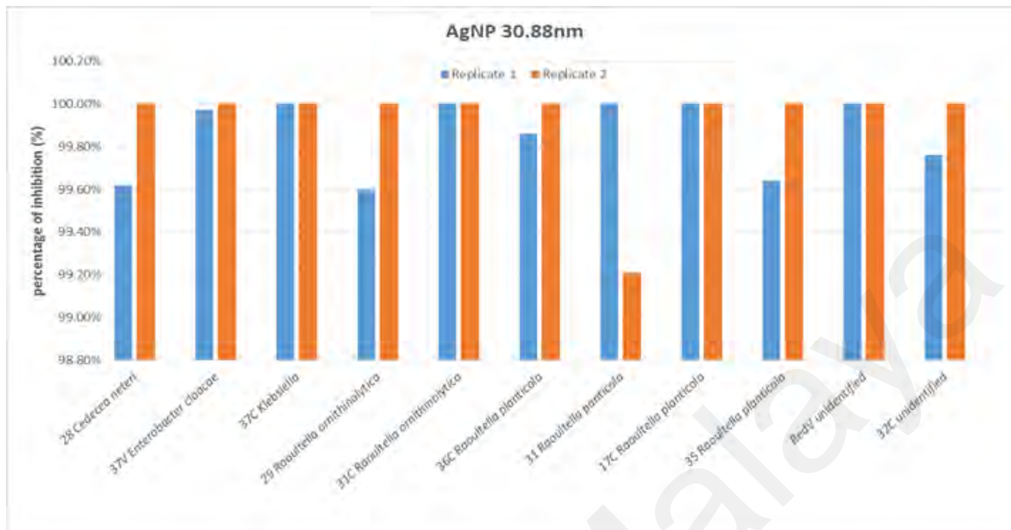

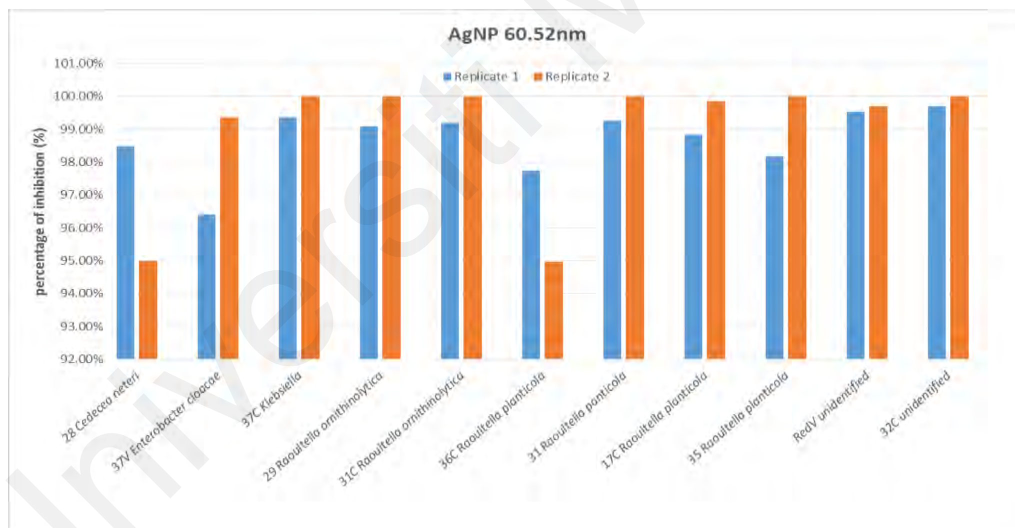

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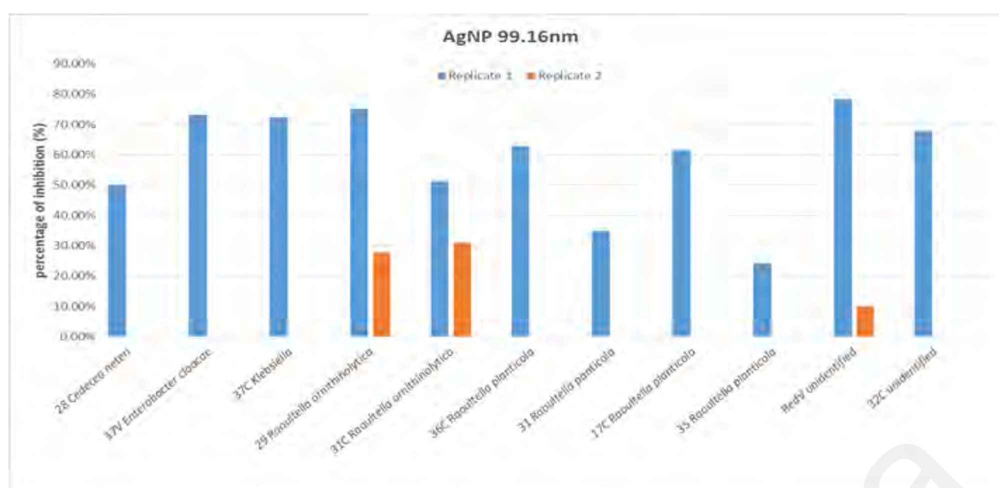

Figure 4.2: Bacterial growth inhibition (%) of the isolated mushroom spoilage bacteria by 50 µg/ml of silver nanoparticles with different diameters (30.88 nm, 60.52 nm, and 99.16 nm).

4.2.2 Minimal Inhibition Concentration of silver nanoparticles against spoilage bacteria of *Pleurotus pulmonarius*

The findings shows that the MIC value for Ag-NPs with the size 30.88 nm was 50 µg/ml for *Cedecea neteri* strain 28, 50 µg/ml for *Enterobacter cloacae* strain 37V, 50 µg/ml for *Klebsiella* sp strain 37C, 50 µg/ml for *Raoultella ornithinolytica* strain 29 and 31C, 25 µg/ml for *Raoultella planticola* strain 36C, 31, 17C and 35, strain RedV was 18.75 µg/ml and Unidentified strain 32C was 50 µg/ml. While the MIC value for the Ag-NPs sized 60.52 nm was 50 µg/ml for *Cedecea neteri* strain 28, 50 µg/ml for *Enterobacter cloacae* strain 37V, 37.5 µg/ml for *Klebsiella* sp strain 37C, 50 µg/ml for *Raoultella ornithinolytica* strain 29, 37.5 µg/ml for *Raoultella ornithinolytica* stain 31C, 25 µg/ml for *Raoultella planticola* strain 36C, 31, 17C, 50 µg/ml for *Raoultella planticola* strain 35, 50 µg/ml for unidentified bacteria strain RedV, 50 µg/ml for unidentified strain 32C. While the MIC value for Ag-NPs sized 99.16nm was >50 µg/ml for all the tested bacteria strains.

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Table 4.2: Minimal inhibitory concentration of each replicate with Ag-NPs sizes 30.88 nm, 60.52 nm, and 99.16 nm.

Ag-NPs	Bacteria	Code	Minimal Inhibitory Concentration (µg/ml)		
			Replicate 1	Replicate 2	Average
Ag-NPs 30.88 nm	*Cedecea neteri*	28	50	50	50
	Enterobacter cloacae	37V	50	50	50
	Klebsiella spp.	37C	50	50	50
	Raoultella ornithinolytica	29	50	50	50
		31C	50	50	50
	Raoultella planticola	36C	25	25	25
		31	25	25	25
		17C	25	25	25
		35	25	25	25
	unidentified	RedV	25	12.5	18.75
		32C	50	50	50
Ag-NPs 60.52 nm	*Cedecea neteri*	28	50	50	50
	Enterobacter cloacae	37V	50	50	50
	Klebsiella spp.	37C	25	50	37.5
	Raoultella ornithinolytica	29	50	50	50
		31C	25	50	37.5
	Raoultella planticola	36C	25	25	25
		31	25	25	25
		17C	25	25	25
		35	50	50	50
	unidentified	RedV	50	50	50
		32C	50	50	50
Ag-NPs 99.18 nm	*Cedecea neteri*	28	>50	>50	>50
	Enterobacter cloacae	37V	>50	>50	>50
	Klebsiella spp.	37C	>50	>50	>50
	Raoultella ornithinolytica	29	>50	>50	>50
		31C	>50	>50	>50
	Raoultella planticola	36C	>50	>50	>50
		31	>50	>50	>50
		17C	>50	>50	>50
		35	>50	>50	>50
	unidentified	RedV	>50	>50	>50
		32C	>50	>50	>50

Figure 4.3 shows the bacteria growth for the bacterial strains tested against Ag-NPs sizes 30.88 nm, 60.52 nm, and 99.16 nm with different dilutions of Ag-NPs of 50 µg/ml,

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25 µg/ml, 12.5 µg/ml, 6.25 µg/ml, 3.125 µg/ml and 1.56 µg/ml. The result showed that the OD readings of the bacterial strains decrease as the concentration of the Ag-NPs increases, indicating the inhibitive capacity increases with the concentration. It was obvious from Figure 4.3 that Ag-NPs 30.88 nm and Ag-NPs 60.52 nm exhibited higher toxicity to the bacterial trains tested as compared to Ag-NPs 99.16 nm. Although Ag-NPs 99.16 nm generally demonstrated increasing growth inhibition capacity with the increase in its concentration, a higher concentration of Ag-NPs beyond 25 µg/mL failed to bring the bacterial density lower (i.e. the inhibition of bacteria growth became stagnant at a concentration above 25 µg/mL).

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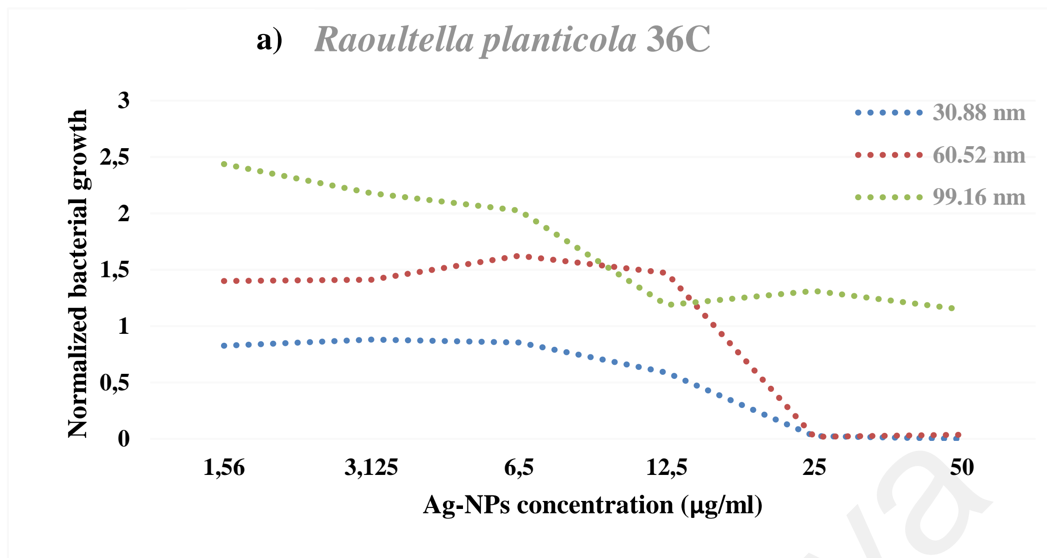

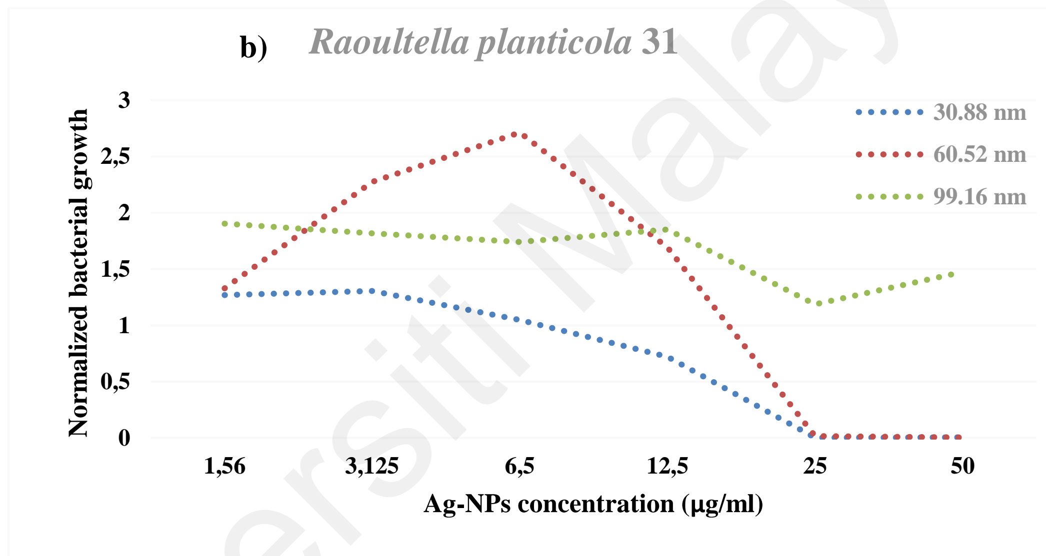

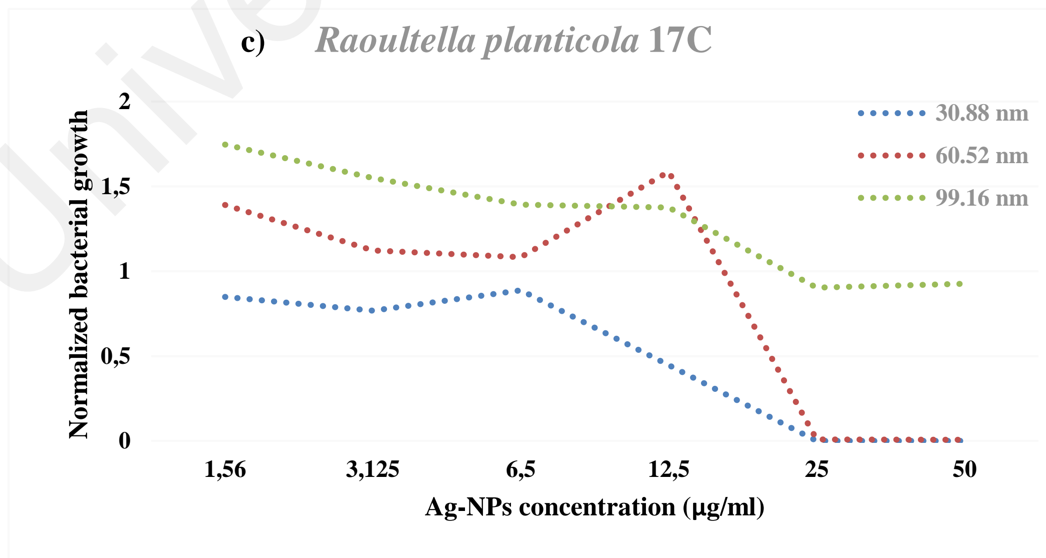

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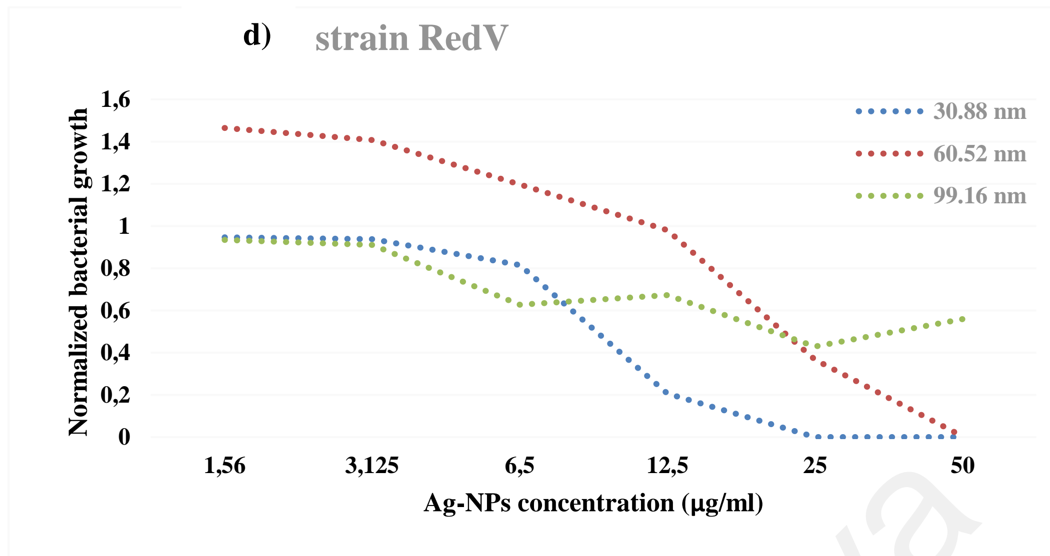

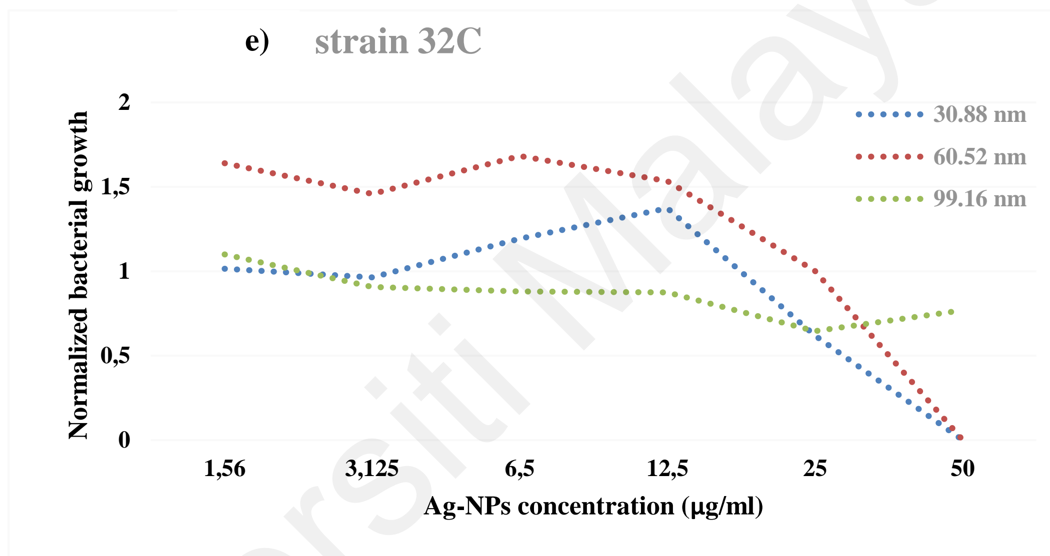

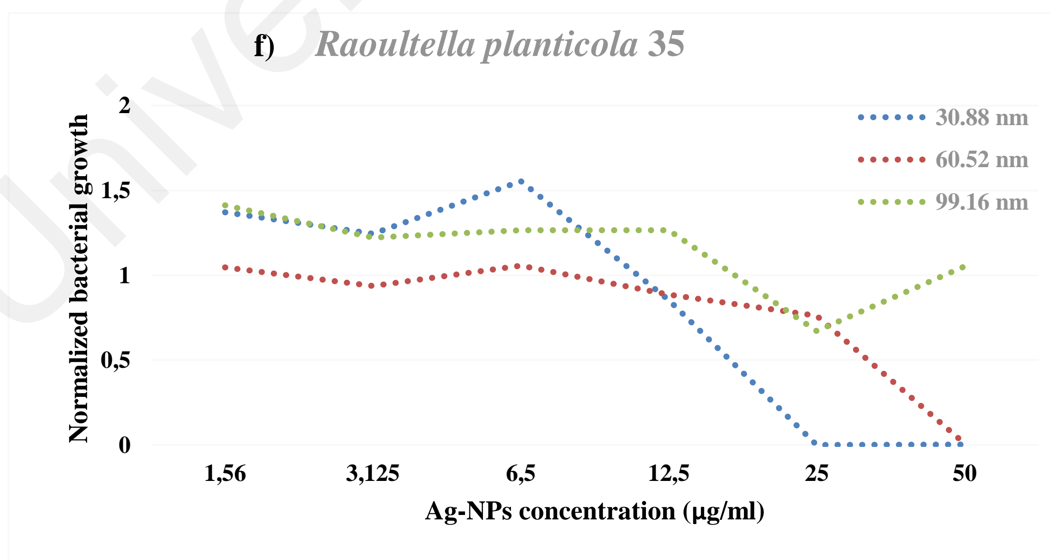

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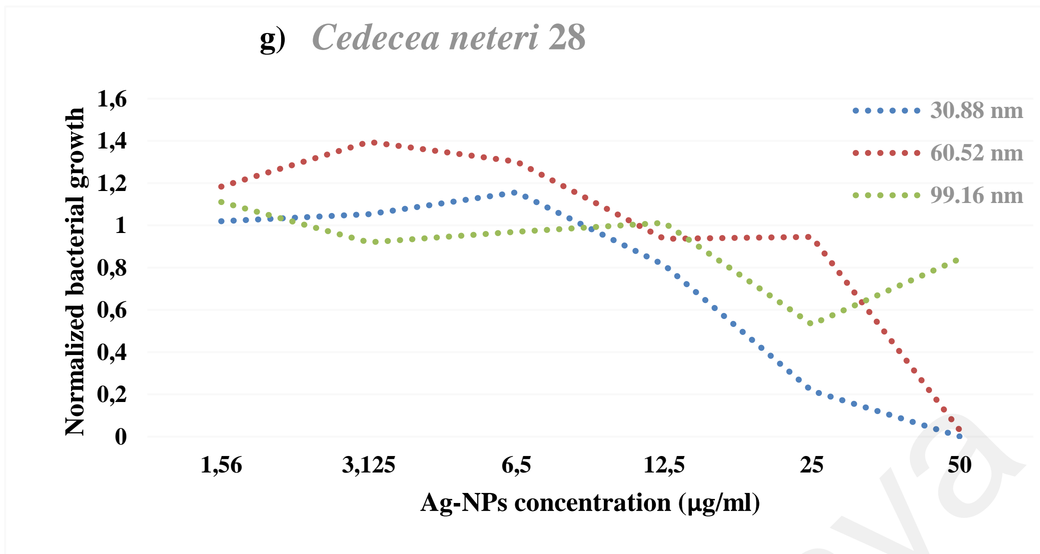

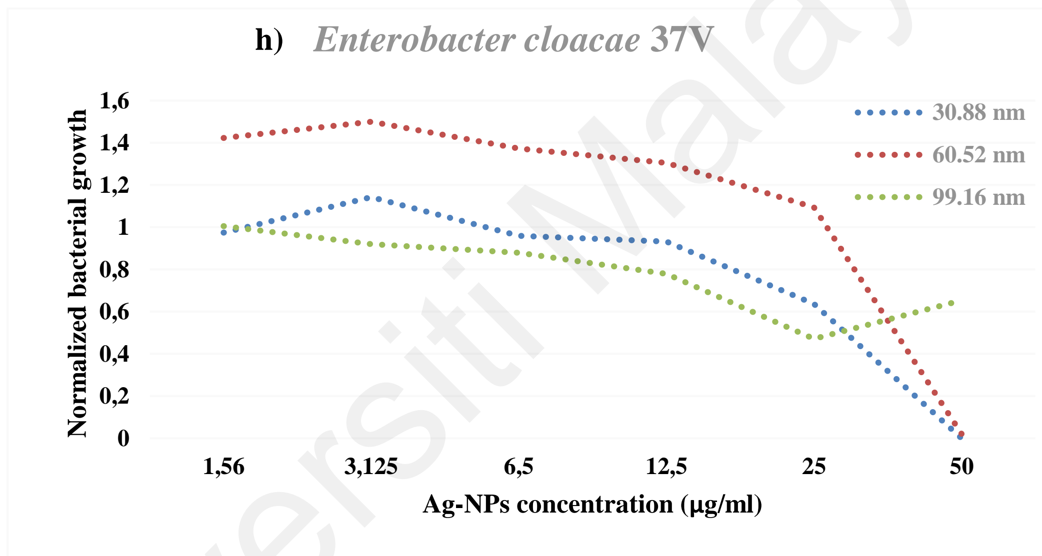

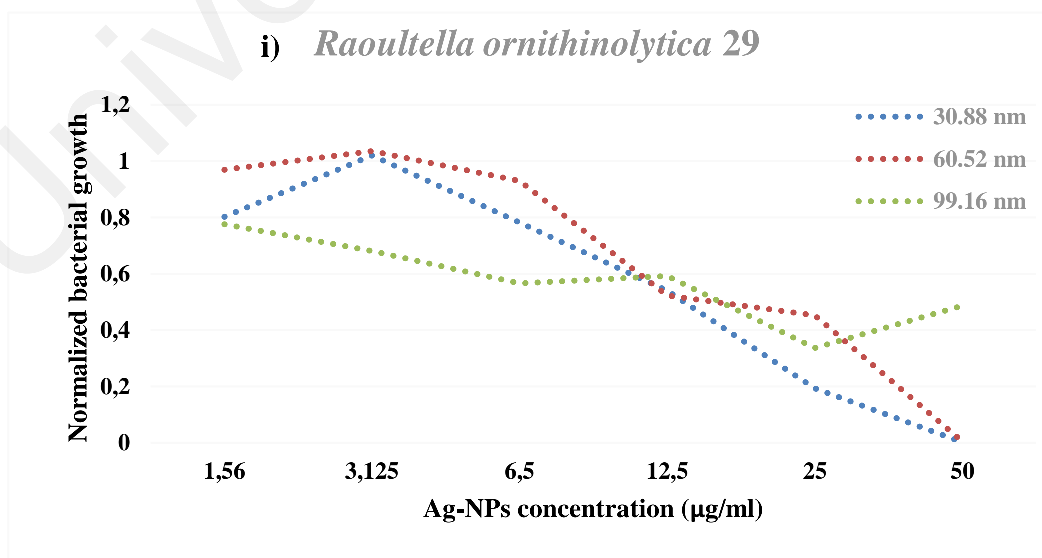

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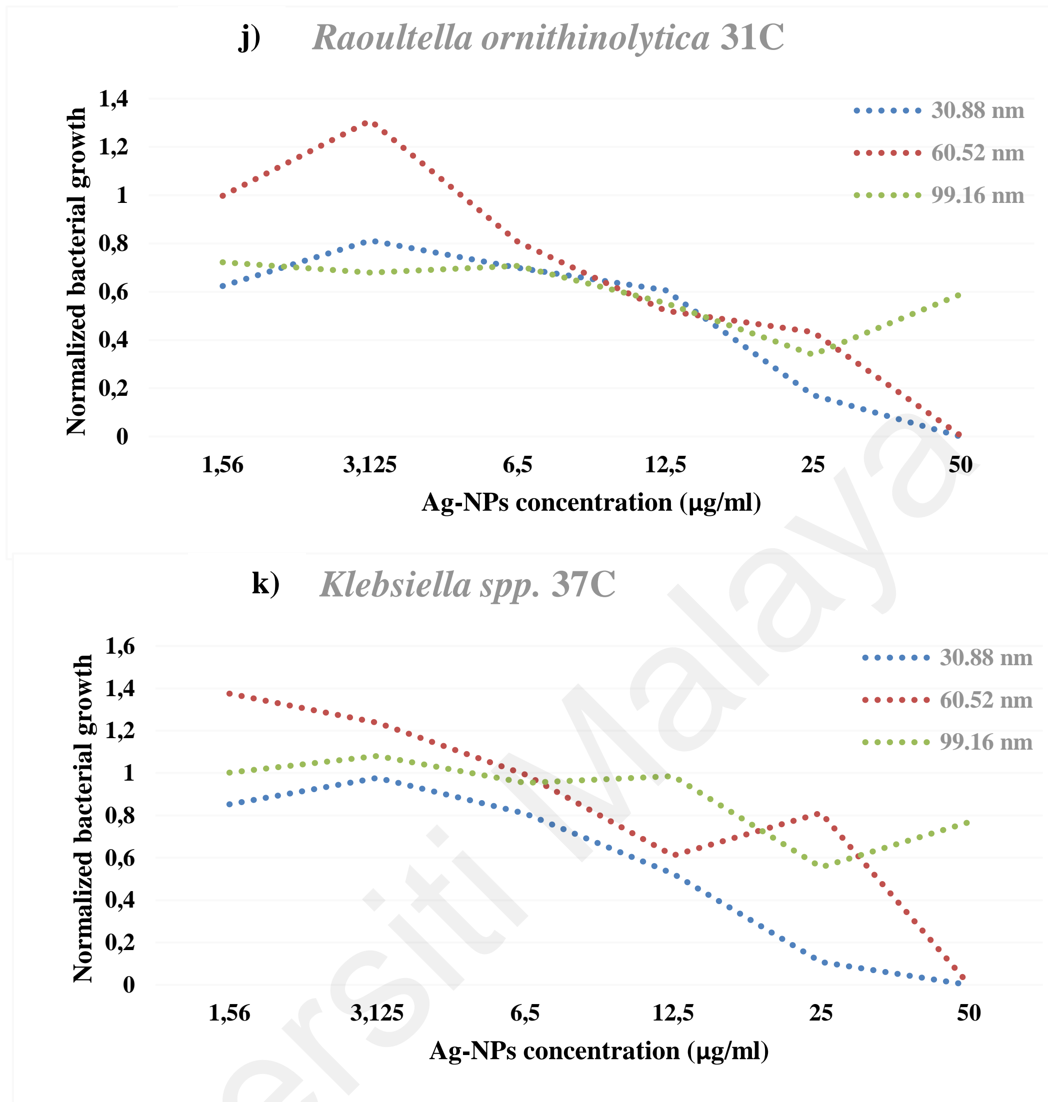

Figure 4.3: The average antimicrobial capacity of Ag-NPs (30.88 nm), Ag-NPs (60.52 nm), Ag-NPs (99.16 nm) against the 11 strains of mushroom spoilage bacteria for Ag-NPs concentration at 50 µg/ml, 25 µg/ml, 12.5 µg/ml, 6.25 µg/ml, 3.125 µg/ml and 1.56 µg/ml. a) *Raoultella planticola* 36C ; b) *Raoultella planticola* 31; c) *Raoultella planticola* 17C; d) strain RedV; e) strain 32C; f) *Raoultella planticola* 35; g) *Cedecea neteri* 28; h) *Enterobacter cloacae* 37V; i) *Raoultella ornithinolytica* 29; j) *Raoultella ornithinolytica* 31C; k) *Klebsiella* spp. 37C.

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CHAPTER 5: DISCUSSION

5.1 Bacteria associated with the spoilage of *Pleurotus pulmonarius*

Raw vegetables and fruits are easily contaminated with spoilage bacteria during pre-harvest, harvest, and post-harvest activities. The main source of contamination on crops could be the untreated wastewater which contains human fecal. Raw and slightly cooked vegetables contaminated by food spoilage bacteria cause food-borne illness to the consumers (Puspanadan et al., 2012).

Pleurotus pulmonarius commonly known as the grey oyster mushroom is rich in vitamins and minerals. Mushrooms contain good supplements of nutrients but are high in water content (Manzi et al., 2004). Non-favorable external factors such as pests, humidity, and environmental temperature can cause mushroom infection in post-harvest mushrooms (Bellettini et al., 2018). *Pleurotus* mushroom is easily spoilt due to microbial attack and the quality normally deteriorates within 3 days after harvest (Xiao et al., 2011), which could be due to high respirational rate and absence of cuticle (Sapata et al., 2009). Proper packaging with antibiotics embedded in the packaging material is needed to prolong the shelf life of mushrooms. Besides proper packaging, it is also important to control the environment of mushroom storage such as low temperature, low humidity, and low carbon dioxide level to overcome the outbreak and contamination (Belletini et al., 2018).

The rotting in mushrooms is also caused by food spoilage bacteria (Pudelko, 2013) *Klebsiella* species, *Raoultella planticola,* and *Enterobacter cloacae* are reported food spoilage bacteria that deteriorate the quality of mushrooms and are usually present in the fruiting bodies of mushrooms (Pudelko, 2013). But these strains are not commonly reported to be infected grey oyster mushrooms. *Enterobacter cloacae, Cedecea neteri,* and *Raoultella ornithinolytica* are reported to contaminate mushrooms during pre and

post-harvest processes (Rossouw & Korsten, 2017). The presence of these mushroom spoilage bacteria can be due to contamination during postharvest handling (Pudelko, 2013). In this study, *Raoultella planticola, Klebsiella* sp. And *Enterobacter cloacae* were identified in the *P. pulmonarius* mushroom fruiting bodies. Enterobacteriaceae presence in the raw vegetables causes foodborne illness to the consumers. Gastrointestinal illness is one of the effects of consuming raw vegetables that are contaminated with spoilage bacteria from the member of *Enterobacteriaceae* (Puspanadan et al., 2012). *Enterobacter cloacae* contamination in ready-to-eat food caused many illnesses in South Africa. *Enterobacter cloacae* can cause many illnesses in humans such as urinary tract infection, gastrointestinal infection, and bacteremia, as these bacteria can cause food spoilage it is recommended to properly cook the mushroom (Nyenie et al., 2012).

A case of multiple organ failure was reported in a patient after consuming *Klebsiella* spp contaminated vegetables (Sabota et al., 1998). *Klebsiella* spp are reported present on African spider herb (*Gleome gynandra*) and dried bush okra (*Corchorus olitorius*), the main source of contamination of these vegetables are due to improper handling and cross-contamination during the processing activities and lack of sanitation in Botswana (Mpuchane and Gashe, 1996). Mpuchane and Gashe (1996) reported that *Klebsiella* spp is found in the intestines of mammals and that indicated that unclean water is contaminated with fecal.

Klebsiella sp contains plasmid-mediated colistin resistance gene, *mcr-1,* and resistance to colistin and causes urinary tract infection (Stoesser et al., 2016; Alves et al., 2014). *Klebsiella* spp. Are commonly found in the intestine, mouth, and skin. This spoilage bacteria induce lung inflammation which causes pneumonia disease. Immunocompromised patients are more prone to be infected by *Klebsiella* spp (Puspanadan et al., 2012). The minimum temperature for the growth of *Klebsiella* spp. It

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Is 8 to 10◦C and the contamination of the spoilage bacteria is higher in the vegetables at hypermarkets compared to the wet market. The reason could be due to the vegetables in the wet market are not stored in a high humidity environment compared to vegetables in the hypermarket. Large surface areas of the vegetables and topographical features of the vegetables and mushrooms make the spoilage bacteria be entrapped and attached to the surface. Vegetables like celery, cabbage, basil, spinach, and lettuce exhibit high relative humidity which could increase the survival rate of *Klebsiella* spp. (Adams et al., 1989). *Klebsiella spp* is present on vegetables and cannot be easily removed by gentle washing and washing with sodium hypochloride solution may reduce the risk of contamination on the vegetables (Garg et al., 1990; Puspanadan et al., 2012).

Raoultella planticola is reported to cause infections in humans such as urinary tract infection, pneumonia, bacteremia, and soft tissue infections (Atici et al., 2018). There is a reported case of conjunctivitis in an infant due to the *R. planticola* infection (Atici et al., 2018). These spoilage bacteria are found mainly on vegetables and mushrooms, soil, water, and insect bodies (Atici et al., 2018). *Raoultella planticola* was previously known as *Klebsiella planticola*. In the year 2001, *Raoultella planticola* was introduced as a new genus in the family *Enterobacteriaceae* (Atici et al., 2018; Puerta-Fernandez et al., 2013).

R. planticola is a rare strain due to a lack of antibiotic susceptibility data and reported studies. The symptoms and the clinical illness caused by *R.planticola* are unknown (Atici et al., 2018; Alsweed et al., 2018; Naganathan & Amin, 2018). *Raoultella ornithinolytica* is considered a blood-borne food spoilage bacteria as it has a similar phenotype as *Klebsiella* spp. (Chun et al., 2015). Other than that, *Raoultella ornithinolytica* and *R. planticola* are also known as histamine producing bacteria that are toxic to scombroid and it is unsafe for human consumption of histamine level scombroid (Sekowska, 2017; Puerta-Fernandez et al., 2013; Lam & Salit, 2014).

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Roultella planticola and *Raoultella ornithinolytica* convert histidine in fish into histamine in poorly cooked fish (Lam & Salit, 2014). *Raoultella ornithinolytica* is reported to tolerate low pH and *Raoultella ornithinolytica* can grow slowly at pH 4 and has optimum growth at a temperature range between 15 to 37°C (Tantasuttikul & Mahakarnchanakul, 2019). Although reported food poisoning of *Raoultella planticola* and *R. ornithinolytica* were mainly on seafood poisoning, food contamination of *Raoultella planticola* is risky for human consumption as it can cause many diseases such as bacteriemia. It is recommended to cook properly as it can destroy foodborne bacteria. *Cedecea neteri* is a rare bacterium that can be found in vegetables and cause bacteremia infection in human beings (Dworkin and Martin, 2006). Oh et al. (2018) reported that these proteobacteria affect the growth of pine mushrooms. *Cedecea neteri* was reported to cause yellow sticky disease and inhibit the elongation of stipes in *Flammulina velutipes* (Yan et al., 2019a; Xiao & Wang, 2019), *Cedecea neteri* infects the stipes of the mushroom causing lesions and dark brown coloration on the stipes of *Pholiota nameko* (Yan et al., 2019b). *Cedecea* sp was also reported to cause blotch disease in *Agaricus brazilensis* (Taparia et al., 2020). The raw consumption of vegetables and mushrooms is unsafe as these bacteria can cause bacteremia (Tan et al., 2017).

Mushroom spoilage bacteria that identified were *Raoultella planticola, Raoultella ornithinolytica, Klebsiella spp, Cedecea neteri,* and *Enterobacter cloacae*. The identification of these bacteria in this study proves that mushroom is prone to microbial attack due to high water content in the fruiting bodies of mushroom and improper handling of mushroom. The identification of these spoilage bacteria-contaminated mushrooms is risky for the consumers and imposes health risks. Proper post-harvest handling of mushrooms is important to minimize the contamination of spoilage bacteria, besides that antimicrobial packaging will reduce the risk of contamination of spoilage bacteria on the mushroom.

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5.2 Antimicrobial Capacity of Ag-NPs against spoilage bacteria of *Pleurotus pulmonarius*

Rajeshkumar (2016) reported that Ag-NPs effectively inhibit *Raoultella planticola* using the disk diffusion method. Maruthai et al. (2017) reported that Ag-NPs killed *Enterobacter* sp by causing leakage of protein and interfering with the DNA replication process of the bacteria species. The minimal amount of spherical-shaped Ag-NPs with a concentration of 20 ng/ml could inhibit the *Enterobacter* sp (Maruthai et al., 2017).

Sulfamethoxazole has antimicrobial properties and actively inhibits both Gram-positive and Gram-negative bacteria (Al-Ahmed et al., 1999). Sulfamethoxazole is used as a positive control in this study as it effectively kills all the spoilage strains. However, sulfamethoxazole is not safe to be used in mushroom packaging since the chemical causes various side effects such as necrolysis, exfoliative dermatitis, Steven-Johnson syndrome, skin allergy, and hematologic effects on children (Asmar et al., 1981).

Ag-NPs is selected for this study as it has a wide range of bactericidal effect against both Gram-positive and Gram-negative bacteria (Abbaszadegan et al., 2015). Small-sized Ag-NPs have a large surface area per volume which increases the bactericidal activity of the nanoparticles (Banach et al., 2016; Hwang et al., 2008). Choi et al (2015) reported that smaller size Ag-NPs possess higher toxicity than the larger size Ag-NPs. There are many killing mechanisms in Ag-NPs such as damaging the bacterial biomolecules, depleting the adenosine triphosphate production triggering the release of positively charged silver ions, and making the strains become reactive oxygen species or superoxide radicals (Hwang et al., 2018). Reactive oxygen species are formed at the cell wall as the positively charged Ag-NPs interact with the negatively charged cell wall of the food spoilage bacteria (Ivask et al., 2014). Ag-NPs increase the production of reactive oxygen species (ROS) which causes damage to DNA destruction and inactivate some other

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essential biomolecules present in the bacteria, (Wang et al., 2017; Das et al., 2017). The generation of free radicals attacks the lipid membrane of the cell and causes rupture of the cell membrane (Kim et al., 2007). The radicals cause lipid peroxidation in the bacteria cell and this inhibits the growth of the bacteria (Madl et al., 2014). Ag-NPs interact with sulphur and phosphorus compounds in the cells causing DNA damage to the cell (Morones et al., 2005). Silver ions released by Ag-NPs disrupt the respiratory function of the bacteria (Bragg and Rainnie, 1974). Silver ions inhibit the site of interaction between flavoproteins and the respiratory chain of the bacteria (Bragg and Rainnie, 1974).

The silver ions inhibit enzyme activity and protein synthesis disrupt ribosomal subunit protein expressions and inhibit the DNA replication process in bacteria (Pal et al., 2007). Chaperones and porins are the non-enzymatic protein binding site of Ag-NPs in the bacterial cell (Wigginton et al., 2010). It is reported that Ag-NPs bind to the thiol groups the functional group of cysteine amino acid, cysteine functions in protein folding and cysteine acts as a catalyst in nucleophilic reaction in the bacteria (Soni et al., 2014). Binding to the thiol groups causes inactivation of the protein and causes DNA to be condensed and denatured (Feng et al., 2000). Other than that, the interaction of silver ion with ribosomal subunits inhibit the expression of enzymes which is involved in the production of Adenosine Triphosphate (ATP) and causes cell destruction.

Silver nanoparticles have a good bactericidal effect against Gram-negative bacteria because of the thin cell wall (El Badawy et al., 2011; Malarkodi et al., 2013). The cell wall of Gram-negative is made up of only about 1-3µm thick polysaccharides layer and about 8nm thick peptidoglycans layer (Slavin et al., 2017). The Gram-negative bacteria cell wall is easily destructed by silver nanoparticles probably because of the absence of a protective layer as in Gram-positive bacteria (Ivask et al., 2014). The electrostatic bonding causes the high uptake of silver ions into the cell wall and the sap vacuole of the

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cell body. The lipopolysaccharides molecules on the Gram-negative bacteria are negatively charged thus uptakes the cations and causing intercellular damage (Vanaja and Annadurai, 2013). This high uptake of silver ions causes stunted growth and formation of free radicals which eventually leads to leakage of bacteria cell walls and membrane (Maruthai et al., 2017; Rajeshkumar, 2016).

Ag-NPs are environmentally friendly and non-toxic to be used as embedded antimicrobial material in mushroom packaging. Thus, using Ag-NPs instead of other antibiotic drugs is recommendable for the packaging of *Pleurotus pulmonarius* (Malarkodi et al., 2013). Ag-NPs are considered innovative technology in prolonging the shelf life of fresh foods (Carbone et al., 2016). Previous studies reported that hydroxypropyl methylcellulose (HPMC) embedded with Ag-NPs films effectively killed bacteria in fresh foods (De Moura et al., 2012). The only concern is the transmission of Ag-NPs transmission to the food, and the migration limit for silver is 10 mg/ml, and below that concentration is considered safe (Carbone et al., 2016). Therefore, embedding these antibiotics compounds in the container or packaging of mushrooms may reduce the spoilage rate of bacteria.

Ag-NPs sized 30.88 nm exhibit the highest antimicrobial capacity because it has the largest surface area per volume ratio compared to 60.52 nm-sized Ag-NPs and 99.16 nm-sized Ag-NPs. It is known that smaller-sized Ag-NPs are more toxic and have a higher bactericidal reaction because they penetrate directly into the cell wall of bacteria, unlike larger sized Ag-NPs which may retain outside of the bacterial cell wall (Sánchez-López et al., 2022). The synergetic combination of Ag^+ and Ag-NPs increases the bactericidal effect of smaller-sized Ag-NPs. It is explained that Gram-negative bacteria are easily killed by Ag-NPs due to the absence of the protective layer on the cell and in the study all the identified mushroom spoilage bacteria were Gram-negative. The growth of

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mushroom spoilage bacteria is delayed at low Ag-NPs concentration because of the bacteriostatic properties of Ag-NPs at low concentration. Ag-NPs at low concentrations exhibit bacteriostatic properties while Ag-NPs at higher concentrations exhibit bactericidal properties, this explains the growth curve of bacterial OD declined at higher concentrations of Ag-NPs (Maiti et al., 2014). At a low concentration of Ag-NPs, the electrostatic interaction between Ag-NPs and mushroom spoilage bacteria cell wall is lower and this is the reason for the growth of mushroom spoilage bacteria at lower Ag-NPs concentration not fully inhibited (Acharya et al., 2018).

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CHAPTER 6: CONCLUSION

The isolated spoilage bacteria from rotten grey oyster mushrooms were *Raoultella planticola, Raoultella ornithinolytica, Klebsiella, Cedecea neteri, Enterobacter cloacae,* and two unidentified strains. *Klebsiella species, Raoultella planticola*, *Raouletella ornithinolytica,* and *Enterobacter cloacae* are known to deteriorate the quality of the mushroom. *Cedecea neteri* strain is not commonly found in *Pleurotus pulmonarius.* There are many reported cases of illness due to food poisoning caused by food spoilage bacteria. The findings showed that Ag-NPs effectively inhibit the growth of the spoilage bacteria. The size of Ag-NPs is an important factor in determining the inhibitory capacity of the Ag-NPs. Ag-NPs with the size of 30.88nm has the highest capacity in inhibiting the growth of the spoilage bacteria tested (99.89±0.21%); the least effective was Ag-NPs 99.16 nm (32.73±30.75%). The MIC value for Ag-NPs with the size of 30.88 nm was 50 µg/ml, and the average MIC value for Ag-NPs with the size of 99.16 nm was >50 µg/ml. Besides the size of the Ag-NPs, the concentration of Ag-NPs is an important factor in determining the inhibitive capability of the Ag-NPs. In this study, the concentration of the spoilage bacteria decreases as the concentration of Ag-NPs increases. Embedding Ag-NPs compound in the mushroom container or packaging may reduce the spoilage rate of bacteria and increases the shelf life of the mushroom. Study on the method of embedding Ag-NPs in containers or packaging; the migration rates of Ag-NPs from the packaging to the mushroom and to study the relationship between migration rates of Ag-NPs to the sizes of Ag-NPs and study the external factors that affect the migration of Ag-NPs such temperature, pH and humidity can be carried out for future studies.

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