UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF GOLD NANOPARTICLES-DNA-BASED SENSOR FOR DETECTION OF Ganoderma boninense

SITI AKHTAR MOHSHIM

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DEVELOPMENT OF GOLD NANOPARTICLES-DNA-BASED SENSOR FOR DETECTION OF *Ganoderma boninense*

By

SITI AKHTAR MOHSHIM

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

November 2021

To my beloved late father, Haji Mohshim Mahamud,

my lovely mother, Hajjah Che Long Hashim,

my wonderful husband, Mohamad Irwan Ahmad,

my love of my life,

Alesya Irdina Mohamad Irwan,

Aaron Iskandar Mohamad Irwan,

Aurora Ilyssa Mohamad Irwan,

Adam Irham Mohamad Irwan,

Adryan Idrees Mohamad Irwan

my supportive siblings, also to Maksu & Paksu

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

DEVELOPMENT OF GOLD-NANOPARTICLES-DNA-BASED SENSOR FOR DETECTION OF *Ganoderma boninense*

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November 2021

Chair : Associate Professor Shahrul Ainliah Alang Ahmad, PhD
Institute : Nanoscience and Nanotechnology

Basal stem rot (BSR) disease caused by the pathogenic fungus known as *Ganoderma boninense* (*G. boninense*) is now an endemic threat to palm health and thus plantation productivity. Currently, there are no effective treatment for BSR, early detection of *G. boninense* is crucial for the control of action. Researchers have developed many scientific discoveries towards detection of the killer fungus. However, the methods are time consuming and required expensive instrumentation and reagents, which some of them need an expertise to run the experiments. These limits bring to the aim of the study, to develop an early detection method employing genomic deoxyribonucleic acid (DNA) without amplification process for the detection of *G. boninense*. The detection of *G. boninense* was conducted based on oligonucleotide probes conjugated to AuNPs. Hybridization of the complementary target DNA was operated in two modes, i.e., (i) colorimetric assay, and (ii) observable features of DNA nano array. In this work, hybridization of targeted DNA was carried out in the presence of two DNA probes conjugated to AuNPs (Probe 1-AuNPs and Probe 2-AuNPs), yielding color changes in solution and visual microscopic changes on nano array. The developed colorimetric sensor utilizing DNA-AuNPs conjugates have a high specificity and ultrasensitive to detect unamplified genomic *G. boninense* DNA as low as 500 zeptogram (zg) which found to be more sensitive compared to the previous research. Besides, the developed method also can detect the stem rot disease as early as stage 1 infection, where there is no visual symptomatic observed on the infected plant. Another mode of detection was performed employing unamplified genomic DNA utilizing the developed Probe 1-AuNPs conjugate with immobilized DNA Probe 2 nano array. The method was able to differentiate and gave a clear illustration under simple light microscope in the positive and negative tested samples with a sensitivity obtained for *G. boninense* DNA of 30 ng. In connection to that, both developed sensors shall be the best early detection methods where it gave better simple understanding to all ages and also suitable for people without scientific background. Therefore, it may consider to be a new alternative early detection method in combating the oil palm pathogen.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

PEMBANGUNAN SENSOR BERASASKAN DNA-NANOZARAH EMAS BAGI PENGESANAN *Ganoderma boninense*

Oleh

SITI AKHTAR MOHSHIM

November 2021

Pengerusi : Professor Madya Shahrul Ainliah Alang Ahmad, PhD
Institut : Nanosains dan Nanoteknologi

Penyakit reput pangkal batang (RPB) yang disebabkan oleh sejenis kulat patogen yang dikenali sebagai *Ganoderma boninense* (*G. boninense*) kini menjadi ancaman endemik terhadap kesihatan dan juga produktiviti tanaman kelapa sawit. Pada ketika ini, masih tiada rawatan yang berkesan untuk membasmi RPB dan oleh sebab itu pengesanan awal *G. boninense* sangat penting untuk tindakan kawalan. Para penyelidik telah membangunkan pelbagai penemuan saintifik bagi mengesan kulat pembunuh ini. Walau bagaimanapun, kaedah-kaedah yang telah dibangunkan agak memakan masa dan memerlukan instrumentasi dan bahan uji yang mahal, yang mana sesetengah daripadanya memerlukan tenaga pakar untuk menjalankan eksperimen yang dibangunkan. Kekangan ini telah mendorong kepada tujuan kajian ini iaitu untuk menghasilkan kaedah pengesanan awal yang baharu melalui penggunakan asid deoksiribonukleik (DNA) genom tanpa melalui proses memperbanyakkan salinan DNA bagi mengesan *G. boninense*. Pengesanan *G. boninense* dilakukan melalui penggunaan prob oligonukleotida yang dihubungkan ke AuNPs. Hibridisasi DNA sasaran pelengkap dikendalikan dalam dua mod, iaitu, (i) ujian kolorimetrik, dan (ii) melalui pemerhatian tatasusunan nano DNA di atas substrat emas. Dalam kajian ini, hibridisasi DNA yang disasarkan dijalankan dengan kehadiran dua prob DNA yang disambungkan ke AuNPs (Prob 1-AuNPs dan Prob 2-AuNPs), yang menghasilkan perubahan warna dalam larutan dan perubahan visual mikroskopik pada tatasusunan nano. Teknik pengesanan melalui perubahan warna yang dibangunkan ini mempunyai tahap pengkhususan dan sensitivity yang tinggi di mana dapat mengesan DNA genomic *G. boninense* tanpa memerlukan proses amplifikasi pada kadar serendah 500 zeptogram (zg) yang mana ianya didapati lebih sensitif berbanding penyelidikan sebelumnya. Selain itu, kaedah yang dibangunkan ini juga dapat mengesan penyakit reput pangkal batang seawal jangkitan pada tahap 1, di mana tiada gejala yang dikenalpasti pada kelapa sawit yang telah dijangkiti. Bagi kaedah pengesanan kedua, hibridisasi DNA genomik yang tidak diamplifikasikan dilaksanakan menggunakan konjugat Prob 1-nanozarah emas dan tatasusunan nano DNA Prob 2 takgerak. Kaedah ini menghasilkan ilustrasi yang jelas apabila dilihat di bawah mikroskop cahaya biasa dalam membezakan sampel positif dan negatif yang diuji di mana kepekaan pengesanan susunan DNA sasaran *G. boninense* dapat dikesan pada

kadar serendah 30 nanogram (ng). Sehubungan itu, penderia yang dibangunkan ini merupakan kaedah pengesanan awal yang terbaik di mana ia memberikan pemahaman mudah kepada semua peringkat umur pengguna dan juga sesuai digunakan oleh individu yang tidak mempunyai latar belakang saintifik. Oleh itu, kaedah pengesanan ini boleh dipertimbangkan sebagai alternatif baharu kepada kaedah pengesanan awal dalam memerangi patogen kelapa sawit.

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"Our fate lives within us…"

Siti Akhtar Mohshim, 2021

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

Shahrul Ainliah binti Alang Ahmad, PhD
Associate Professor
Department of Chemistry
Faculty of Sciences
Universiti Putra Malaysia
(Chairman)

Nor Azah binti Yusof, PhD
Professor
Department of Chemistry
Faculty of Sciences
Universiti Putra Malaysia
(Member)

Muhammad Zamharir bin Ahmad, PhD
Director of MARDI Perlis
Malaysian Agricultural Research and Development Institute (MARDI)
(Member)

ZALILAH MOHD SHARIFF, PhD
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School of Graduate Studies
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Signature: _____
Shahrul Ainliah binti Alang Ahmad, PhD
Associate Professor
Committee: Chairman

Signature: _____
Nor Azah binti Yusof, PhD
Professor
Committee: Member

Signature: _____
Muhammad Zamharir bin Ahmad, PhD
Committee: Member

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2D	2-dimensional
3D	3-dimensional
5.8S	5.8 subunits
8-MOP	8-methoxypsoralen
18S	18 subunits
28S	28 subunits
A	Adenine
A_{280}	Absorbance value at 280 nm
A_{260}	Absorbance value at 260 nm
AG	Aktiengesellschaft or stock corporation
ANN	Artificial neural network
Apo E	*Apolipoprotein E* gene
Au	Gold
AuNP(s)	Gold nanoparticle(s)
Au-S	Gold-Sulfide
BLAST®	Basic Local Alignment Search Tool®
bp	Base pair
BSR	Basal stem rot
C	Cytosine
CCCVd	Coconut cadang-cadang viroid
cDNA	Complementary DNA
CNV	Copy number variant(s)
Co.	Company
Corp.	Corporation

CPO	Crude palm oil
CT	Computer tomography
Cy5	Cyanine 5 fluorescent labeled
Dev	Deviation
DI	Deionized
DLS	Dynamic light scattering
DNA(s)	Deoxyribonucleic acid(s)
DNA-AuNPs	DNA-gold nanoparticles
DOSM	Department of Statistics Malaysia
DPO	Dual priming oligonucleotide
dsDNA	Double-stranded DNA
EDTA	Ethylenediaminetetraacetic acid
e.g.	Example
ELISA	Enzyme-linked immunosorbent assay
E-Nose	Electronic nose
ERM	European Reference Material
Err	Error
et al.	And others
etc.	And others
FASTA	A text-based format for representing either nucleotide sequences or amino acid (protein) sequences, in which nucleotides or amino acids are represented using single-letter codes
FFB	Fresh fruit bunch
FRET	Fluorescence resonance energy transfer
G	Guanine
G.	*Ganoderma*

G4	Guanine-quadraplex
G-C	Guanine-cytosine content
GanET	Reverse primer for identification of *Ganoderma boninense*
GSM	*Ganoderma* selective medium
HPLC	High performance liquid chromatography
i.e.	That is
IDT	Integrated DNA Technologies
Inc.	Incorporated
in situ	On site/on position/in place where the event take place
in vitro	In "test tube" experiment
IP	Isoelectric point
IRMM	Institute for Reference Materials and Measurements
ITS	Internal transcribed spacer
ITS1	Internal transcribed spacer 1
ITS2	Internal transcribed spacer 2
ITS3	Forward primer for identification of *Ganoderma boninense*
Jacq.	Jacquin
JRC	Joint Research Centre
LAMP	Loop-mediated isothermal amplification
LC	Limited company
Llc.	Limited liability company
LOD	Limit of detection
LSPR	Localized surface plasmon resonance
Ltd.	Limited
MARDI	Malaysian Agricultural Research and Development Institute
MEA	Malt extract agar

MDNA	Mismatch DNA
MMP	Magnetic microparticles
MPOB	Malaysian Palm Oil Board
Mr.	Mister
NCBI	National Center for Biotechnology Information
NDNA	Non-complementary DNA
NIFOR	The Nigerian Institute for Oil Palm Research
Nil	Nothing/Unavailable
NNLS	Non-negative least squares
pAb(s)	Polyclonal antibody(s)
PALS	Phase-analysis light scattering
PB	Phosphate buffer
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PCR-RFLP	PCR-Restriction length polymorphism
PDA	Potato dextrose agar
PGY	Peptone yeast glucose
PGY-PDA	Peptone glucose yeast-potato dextrose agar
pH	Numerical/logarithmic scale to specify acidity or alkalinity
PKO	Palm kernel oil
POC	Point-of-care
PPL	Polymer pen lithography
PPO	Processed palm oil
Probe 1-AuNPs	Probe 1-gold nanoparticles
Probe 2-AuNPs	Probe 2-gold nanoparticles

Pte.	Private
Q1	Quarter 1
QD	Quantum dot
RFLP	Restriction fragment length polymorphisms
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RT	Room temperature
RT-PCR	Real-time PCR
S1	Round-up Ready™ soybean sample
SCMV	Sugar cane mosaic virus
SEM	Scanning electron microscope
sp.	Species
spp.	Several species
SPPR	Surface plasmon polariton resonance
SPR	Surface plasmon resonance
ssDNA	Single-stranded DNA
Std	Standard
T	Thymine
TAE	Tris-acetate-EDTA
taxid	Taxonomy identifier
TBE	Tris-borate-EDTA
TCEP	Tris(2-carboxyethyl)phosphine
TDNA	Target DNA
TEM	Transmission electron microscope/microscopy
UPM	Universiti Putra Malaysia

USA	United States of America
USR	Upper stem rot
UV	Ultraviolet
UV-vis	Ultraviolet-visible

LIST OF SYMBOLS AND UNITS

ºC	Degree Celsius
λ	Lambda
λ max	Maximum peak absorption
%	Percent
≥	Greater or equal to
≤	Less or equal to
±	More or less
+	Positive charge
-	Negative charge, or minus, or to
/	Or
~	Approximately
:	Ratio
™	Trademark
®	Registered trademark
×	Time(s)
→	To
µL	Microliter(s)
µM	Micromolar
µm	Micrometer(s) or micron
ζ	Zeta
ζP	Zeta potential
cm	Centimeter(s)
g	Gram(s)

h	Hour(s)
M	Molar
MΩ cm-1	Megaohms per centimeter resistivity unit
M-1cm-1	Molar absorptivity coefficient unit
mg	Milligram(s)
Ha	Hectares
L	Litre
MHa	Million hectares
min	Minute(s)
mL	Milliliter(s)
mm	Millimeter(s)
mM	Millimolar
MMt	Million metric tons
MT	Metric tons
Mt	Million tons
MV	Millivolt(s)
ng	Nanogram(s)
ng/µL	Nanogram(s) per microlitre(s)
nm	Nanometer(s)
nM	Nanomolar
pmol	Picomol
RM	Ringgit Malaysia
RPM	Revolution per minute
s	Second(s)
t	Ton(s)

V	Volt(s)
v/v	Volume per volume
yg/µL	Yoctogram(s) per microlitre
zg	Zeptogram

CHAPTER 1

INTRODUCTION

1.1 Background

Palm oil is an edible vegetable oil that has been found in almost all of our daily products such as consumer foods, livestock's foods, confectioneries and cosmetics (Voora et al., 2019). A few years ago, palm oil has also been used as biofuels in response to the sustainable agricultural campaign and towards global concern for a greener world (Mykhaylova, 2018; Supramaniam, 2016). Consumption of the palm oil is widely used around the world especially in Malaysia and is claimed as one of the cheapest edible and versatile oil compared to other edible oils such as olive oil, sunflower oil, canola oil, etc. (Hirschmann, 2020). Oil palm (*Elaeis guineensis* Jacq.) is one of the major agricultural commodity for Malaysian trade sector (Din, 2018; Ministry of Plantation Industries and Commodities, 2020). A statistic report provided by Statistica Inc., showed that Malaysia dominates the world's palm oil industry (Hirschmann, 2020) where the export volume of Malaysian palm oil for quarter 1 (Q1) in 2020 shows a reduction to 12.13 Mt compared to Q1 in 2019 (13.83 Mt). This situation shows that the export volume of Malaysian palm oil within a year has decreased by 12.3% (Ministry of Plantation Industries and Commodities, 2020). Due to the increasing of global demand on the palm oil, Malaysia is also experiencing an inadequate for domestic supply.

The reductions in palm oil export as well as domestic needs is attributable to the stem rot diseases (basal stem rot and upper stem rot) on the oil palm trees, which are infected by a fungus named *Ganoderma boninense* (*G. boninense*) (A. Roslan and A. S. Idris, 2012; Flood et al., 2000). The industries and also the palm oil producing countries especially Malaysia, suffer a huge losses due to the shrinkage of the export and foreign exchanges (Hushiarian, 2015). The value of losses could be greater in the future due to the difficulties in identifying the diseases at early stage. The damages caused serious impact to the smallholders as well as to the oil-palm based industries (A. Roslan and A. S. Idris, 2012). It is necessity to overcome these problems where hitherto been no cure for the diseases. To date, there are no reported solution in eradicating this stem rot diseases. To overcome this issue, an early diagnostics method which is specific to the main causal of stem rot diseases yet also sensitive must be developed. It is required to have an early detection to identify suffered oil palm trees as early as possible in a way to save the entire palms of the plantation thus able to rescue the smallholders and the oil palm industries.

Several studies either by biological, chemical, and physical methods were attempted to overcome the absence of early detection method on *Ganoderma sp.* Nevertheless, some of the techniques that previously reported are not based on analytical methods but only by traditional symptoms observation. These methods require very frequent monitoring by workers to avoid infection in the entire plantation area. Another observation method is through fungal cultural studies on *Ganoderma* selective medium (GSM) (Darus & Abu Seman, 1992). Due to slow progress, both methods are not suitable to be labeled as early detection methods and the difficulties to cover a large-scale applications became a matter

of course (L. Rajendran et al., 2009). With rapid and advances in the research area, various types of biosensors have been developed through signal transduction such as, by colorimetric (Natarajan et al., 1986), electrochemical (Dutse et al., 2012; Hushiarian, 2015), and optical (Ahmadi et al., 2017; Alexander et al., 2014) methods which applied to detect *G. boninense*. The combination of substances such as antibodies, cells, enzymes, and deoxyribonucleic acid (DNA) together with the developed technologies can result to a more selective biosensor method in identifying *G. boninense*. Molecular DNA- and immunological-based detection methods such as, polymerase chain reaction (PCR) (Abu Seman et al., 2003, 2010; Mandal et al., 2014; Panchal & Bridge, 2005) and enzyme-linked immunosorbent assay (ELISA) (Madihah et al., 2014; Utomo et al., 1997) were reported as early detection methods. But on the other hand, most of the methods require highly skilled labor and must be operated in the laboratory.

The availability of nucleic acids through synthetic synthesis which is able to specifically target analytes and produce specific binding that exhibit a simple helical ribbon formation to a complex structures such as, hairpin and guanine-quadraplex (G4) together with their relative chemical properties, making them more preferred than antibodies and other biological analytes (Mondal et al., 2018). Nucleic acid which is known as DNA has become an interesting biomolecule due to these advantages. In addition, the easiness to operate, high in target affinity, easy to label, as well as low-cost production, makes it an attractive analyte for the development of biodiagnostic methods (Famulok & Mayer, 2011). Besides the DNA, gold nanoparticle (AuNP) has been known as a metal nanomaterial that offers as colorimetric-based sensing in foods (Draz et al., 2018), clinical (S.-H. Chen et al., 2009), environmental (Amanulla et al., 2017), and agricultural sectors (Baetsen-Young et al., 2018). Gold nanoparticles (AuNPs) with combination of biological substances are an ideal pair in biosensor developments. The stability of AuNPs increases after functionalization with nucleic acids and thus makes them suitable in producing sensing tools. Therefore, development of colorimetric methods using DNA functionalized with AuNPs could be an interesting new alternative detection system in targeting the *G. boninense*. Additionally, the high target selectivity and ultrasensitivity from the deployment of DNA-AuNPs conjugates in many biosensor applications has resulted in extraordinary and spectacular potential for early detection of *G. boninense*.

1.2 Motivations

1.2.1 Problem statements

G. boninense is a pathogen that causes to basal stem rot (BSR) and upper stem rot (USR) of oil palm, eventually reducing the yield of the crop. Until now, there is no effective treatment for this fungus. While waiting for the emergence of the fungal exterminator to exist, early detection method is needed to save the oil palm plantations and industries as well as the economy of the producing countries. Researchers around the world come with innovative ideas in developing sensors to detect the major cause of the deadly disease for oil palm, which is known as *G. boninense* fungus. However, most of the published or commercial techniques such as, GSM which involves a time-consuming traditional culturing method and observation (Darus & Abu Seman, 1992), a sensitive ELISA method (Madihah et al., 2014; Utomo et al., 1997) which might create a cross-reactivity

results due to unspecific antibodies developed from animals source (Lau, 2016), PCR (Abu Seman et al., 2003; Mandal et al., 2014; Panchal & Bridge, 2005) that famously known as very selective and sensitive method but yet the method is shadowed by the utilization of sophisticated equipment and the needed of expertise. These methods clearly show that there are limitations as well as the need of high-trained personnel with expensive instrumentation end up led to expensive costing in order to detect the fungus. Since 1996, 5 – 6% healthy-looking oil palms were reported infected with the fungus (Ariffin et al., 1996). The early stage (level 1) *Ganoderma* infected oil palms are asymptomatic, thus made it difficult to define the oil palm condition (Ibrahim et al., 2020). Previous studies indicate that the earliest symptoms can be differentiated between the healthy and level 1 infected oil palm trees using a high resolution hyperspectral remote sensing (Shafri et al., 2011). However, the sensitivity detection through the method was unreported. The advanced biosensors such as, tomography (J. Abdullah et al., 2013; Ishaq et al., 2014), electronic nose (E-Nose) (Kresnawaty et al., 2020; Markom et al., 2009), ergosterol analysis (Muniroh et al., 2014), infrared spectroscopy (Alexander et al., 2014; Noor Azmi et al., 2020), etc. can provide rapid and robust in pathogen detection but not a simple yet sensitive detection methods.

1.2.2 Objectives of the study

The aim of this thesis was to develop a simple, specific, and sensitive gold nanoparticles-DNA-based sensors for the determination of *G. boninense*. The proposed sensing methods were aimed to be conducted in two modes, which are:

- colorimetric assay in combination with UV-visible (UV-vis) readouts
- detection based on dot features of nano array appearance with optical microscope.

In order to achieve the goal, the specific technical objectives are:

1. To design two DNA probes (Probe 1 and Probe 2) by selecting specific sequence from ITS gene of *G. boninense* genome.
2. To produce, optimize and characterize DNA-AuNPs conjugates (Probe 1-AuNPs and Probe 2-AuNPs) as hybridization platform for colorimetric sensor.
3. To investigate hybridization systems in assay solution and on nano array employing the produced DNA-AuNPs conjugates with synthetic DNA and fragmented genomic DNA samples.

1.2.3 Practical relevance of the study

This study is motivated by the need in designing a novel selective and sensitive DNA biosensor at point-of-care (POC) without amplification of DNA where complex protocol, equipment and biological sample purification are eliminated. Selected DNA probes conjugated to AuNPs were employed to detect DNA target sequence (35 bases) which exist in *G. boninense*. The color of the assay is expected to change (from reddish to purplish color) through DNA hybridization. A localized surface plasmon resonance (LSPR) readout from the assay could be carried out to show quantitative results due to aggregation of AuNPs in the presence of complementary sequence. The hybridization of target sequence in synthetic and genomic DNA on the nano array surface could be observed under a simple light microscope, which offers a simple, easy-to-use and portable sensor for on-site monitoring.

1.2.4 Importance and significant contribution

The arose problem statements specify to the question; what is important or influencing actions to be taken in order to eradicate the disease? Herein, the author will discuss the findings of the study which will contribute to the benefit of consumers and industries related to the oil palm products. The necessity of this study is to develop a new early detection method in detecting *G. boninense* with a simple application but yet still selective and sensitive. The study is also to create a POC diagnostic concerning to the loss of crops due to basal stem rot (BSR) disease which affecting the economics of palm oil producing countries. Thus, the developed sensor will lead towards eliminating the disease faster than the current available approaches.

1.3 Thesis structure overview

This thesis is composed of five chapters with references and appendices. Each chapter will be discussed in detail as follows:

1. Chapter 1 presents general overview of the oil palm and BSR disease. Research motivation, objectives and significant relevance of this study were also discussed in this chapter.

2. Chapter 2 presents a literature review of the oil palm, economic impact, BSR disease outbreak and the pathogens that trigger the BSR disease and infection to the oil palm. This chapter also discussed about current available detection methods and disease management strategies. The chapter also includes the development of colorimetric detection targeting the complementary DNA utilizing DNA probes functionalized AuNPs and hybridization mechanism. In addition, the properties and functionalization of DNA probes to AuNPs were also reviewed.

3. Chapter 3 describes the experimental details from selecting and analyzing the DNA probe sequences, real sample collections, pure culture isolation and identification. The conjugation of DNA-AuNPs, optimizations and characterizations of the functionalized nanoparticles properties are presented. Details of hybridization technique based on color changes and the quantitative LSPR study are described.

4. Chapter 4 presents the experimental results obtained from the study. The specificity and sensitivity level depicting the limit of detection (LOD) of the developed sensor are discussed in this chapter employing two methods i. e., hybridization in assay solution, as well as on nano array.

5. Chapter 5 presents a conclusion and summary of the present study. Some recommendations for possible future works are also included.

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