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The Effect of Zinc Oxide and Titanium Dioxide Nanoparticles on Extraction, Purification, Genomic DNA Amplification and Transformation in Some Gram Negative Bacteria

A Dissertation

Submitted to the Council of College of Science, Al-Nahrain University, as a Partial Fulfillment of the Requirements for the Degree of Doctorate of Philosophy in Science, Biotechnology

By

Muntaha Razzaq Ibrahim

B.Sc. in Biology/ University of Baghdad/ 1999

M.Sc. in Biotechnology/ Al-Nahrain University/ 2006

Supervised By

Professor Dr.

Dr. Majed Hussein Al-Jelawi

October 2014

Professor Dr.

Dr. Ayad M.A. Fadhil

Thee Al- Hija 1435

Supervisors Certification

We, certify that this dissertation entitled "**The Effect of Zinc Oxide and Titanium Dioxide Nanoparticles on Extraction, Purification, Genomic DNA Amplification and Transformation in Some Gram Negative Bacteria** was prepared by "**Muntaha Razzaq Ibrahim** " under our supervision at the College of Science/Al-Nahrain University as a partial fulfillment of the requirements for the Degree of Doctorate of Philosophy in Science (Biotechnology).

Signature:

Name: **Dr. Majed H. Al-Jelawi**

Scientific Degree: Proffesor

Date: / / 2015

Signature:

Name: **Dr. Ayad M.A. Fadhil**

Scientific Degree: Proffesor

Date: / / 2015

In view of available recommendations, I forward this dissertation for debate by examining Committee.

Signature:

Name: Dr. Hameed M. Jasim

Scientific Degree: Professor

Title: Head of Biotechnology Department

Date: / / 2015

Committee Certification

We, the examining committee certify that we have read this dissertation entitled "**The Effect of Zinc Oxide and Titanium Dioxide Nanoparticles on Extraction, Purification, Genomic DNA Amplification and Transformation in Some Gram Negative Bacteria** "and examined the student " **Muntaha Razzaq Ibrahim''** in its contents and that in our opinion, it is accepted for the Degree of Doctorate of Philosophy in Science (Biotechnology).

Signature:
Name: Dr. Salih A. Whaib
Scientific Degree: Professor
Date: / / 2015
(Chairman)

Signature:
Name: Dr. Rehab S. Ramadhan
Scientific Degree: Assistant Proffesor
Date: / / 2015
(Member)

Signature:
Name: Dr. Abdul Kareem Al Kazaz
Scientific Degree: Proffesor
Date: / / 2015
(Member)

Signature:
Name: Dr. Mohammed Faraj Al Marjani
Scientific Degree: Assistant Proffesor
Date: / / 2015
(Member)

Signature:
Name: Dr. Adawiya J. Haider
Scientific Degree: Proffesor
Date: / / 2015
(Member)

Signature:
Name: Dr. Majed H. Al-Jelawi
Scientific Degree: Professor
Date: / / 2015
(Member/ Supervisor)

Signature:
Name: Dr.Ayad M.A.Fadhil.
Scientific Degree: Professor
Date: / / 2015
(Member/ Supervisor)

I, here by certify upon the decision of the examining committee.
Signature:
Name: Dr. Hadi M. A. Abood
Scientific Degree: Assistant Professor
Title: Dean of the Science College
Date: / / 2015

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muntaha

Summary

This study aimed to investigate the effect of using ZnO and TiO_2 nanoparticles on some molecular techniques in order to improve them.

The nanoparticles used in this study were characterized using UV/vis spectrophotometer and transmission electron microscope. Results showed that both nanoparticles were highly purified, their shape was spherical and the average diameter was about 100 nm and 50 nm for TiO_2 and ZnO respectively.

The effect of nanoparticles (TiO_2 and ZnO) on bacterial growth was determined. Results illustrated that nanoparticles had antibacterial effect on gram negative bacteria (*Escherichia coli* HB 101, *Pseudomonas aeruginos*a). The best antibacterial effect was at concentration of 4 mg\ml for TiO_2 and 3 mg/ml for ZnO.

The minimum inhibitory concentration (MIC) of ZnO on *E. coli* HB 101, *E. coli* MM294 and *P. aeruginosa* was 0.05, 0.05 and 0.5 mg/ml respectively. While the MIC of TiO_2 on these bacteria was 0.35 mg/ml.

Quality and quantity of DNA isolated by boiling, alkali lysis and salting out methods in presence of nanoparticles were investigated. It was found that ZnO gave a good enhancement of DNA quality and quantity of *E. coli* and *P. aeruginosa*. While TiO_2 led to enhancement of DNA quality for both bacteria by all DNA extraction methods. However, TiO_2 caused enhancement of DNA quantity of both bacteria when used with salting out method only.

Amplifications of *lacZ* gene of *E. coli* and 16S rRNA gene of *P. aeruginosa* were performed in presence of nanoparticles. It was found

that ZnO (0.4 mg/ml) was highly enhanced PCR products for both genes. The purity of these products was improved also when using both nanoparticles. Results declared that there is about 50 % reduction on over all PCR reaction time by using both nanoparticles.

According to these results, it can be concluded that ZnO might improve the specificity of primers, enhance thermal conductivity of fluid and inhibit restriction enzymes.

In order to study the effect of nanoparticles on the transformation, *E. coli* MM294 was transformed with genomic DNA of *E. coli* HB 101 and *P. aeruginosa*. Result indicated that 0.4 mg/ml of ZnO and 0.2 mg/ml of TiO_2 were enhanced *E. coli* MM294 transforments.

Results reported also that transformation frequency was more increased when $MgCl_2$-$CaCl_2$ solution replaced by nanoparticles solution, which also reduced the chemical components. Transformation frequencies were increased for about 1.3 and 2.1 folds with TiO_2 and ZnO respectively, which means that ZnO gave the best results.

To study the effect of nanoparticles on the conjugation. Conjugation experiments were performed between *E. coli* Hb 101 and *P. aeruginosa* in presence of nanoparticles. Results showed that there was no effect of any concentration of nanoparticles on conjugation, in which transconjugants still the same with and without using nanoparticles.

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List of Abbreviation

Abbreviation	Full Name
AML	Acute myelogenous leukemia
ATCC	American Type Culture Collection
AU	Absorption unit
Au NP	Gold nanoparticles
bp	Base pair
CNTs	Carbon nanotubes
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide tri phosphate
DDH2O	Deionised Distilled water
D.W	Distilled water
DNase	Deoxyribonuclease
ELISA	Enzyme-linked immuonoabsorbent assay
EDTA	Ethylene diamine tetra acetic acid
IARC	International Agency for Research on Cancer
Kb	Kilo base
LB	Luria - Bertani
MEMS	Microelectromechanical systems
µl	Microliter
µg	Microgram
MIC	Minimum Inhibition Concentration
MWNT	Multi Wall Nano Tube
NCCLs	National committe for clinical laboratory standards
NPs	Nanoparticals
NEMS	Nano Electro Mechanical Systems
nm	Nanometer
PCR	Polymerase Chain Reaction
pH	Power of Hydrogen
pmol	Picomole
PNPs	Polymeric nanoparticles
RNA	Ribonucleic acid

rRNA	Ribosomal Ribonucleic Acid
rpm	Rotation per minute
SDS	Sodium Dodecyl Sulphate
Spp.	Species
SLN	Solid lipid nanoparticles
SWNTs	Single-wall nanotubes
TiO_2	Titanium dioxide
TBE	Tris-Borate-EDTA
TEM	Transmission Electron Microscope
Tris-base	Tris (hydroxymethyl)aminomethane base
UV	Ultra Violet
VIS	Visible spectrophotometer
ZnO	Zinc oxid

Chapter one

Introduction
And
Literature review

1. Introduction and Literature Review

1.1 Introduction

The field of nanotechnology is one of the most active areas of research in modern material sciences. Nanotechnology is a field that is developing day by day, making an impact in all spheres of human life and creating a growing sense of excitement in life sciences especially biomedical devices and biotechnology.

Nanoparticles exhibit completely new or improved properties based on specific characteristics such as size, distribution and morphology. Nanoparticles of noble metals, such as gold, silver, platinum and zinc oxide are widely applied in products that directly come in contact with the human body, such as detergent, cosmetic products and toothpaste besides medical and pharmaceutical applications (Birhanli *et al.,* 2014).

In the field of medicine, nanoparticles are being explored extensively because of their size dependant chemical and physical properties. The size of nanoparticles is similar to that of most biological molecules and structures. This makes them an interesting candidate for application in both *in vivo* and *in vitro* biomedical research. The result of their integration in the field of medicine has led to their application mainly in targeted drug delivery, imaging, sensing, and artificial implants. Another interesting avenue for their exploration in medicine is their use as antimicrobials to target highly pathogenic and drug resistant microbes (Ravishankar and Jamuna, 2011)

Nanobiotechnology applications could potentially be quite diverse, from building faster computers to finding cancerous tumors that are still invisible to the human eye (Doudricck, 2013).

One of the main reasons nanobiotechnology holds so much promise is that it operates at the biological size scale. Biological molecules (such as enzymes, receptors, DNA), microorganisms and individual cells in our bodies are all nano-sized. Engineered ultrasmall particles that are made in the exact size needed to perform specific tasks, such as drug release in particular locations in the body, drug delivery into the blood stream, or to pin- point malfunctioning tissues (cancerous tissue, for example), are examples of the new medical discipline termed 'nanomedicine' (Constance, 2013).

The rapidly growing field of molecular biology and biotechnology has a tremendous need for quick, simple, robust and high-throughput procedures for extraction of DNA from diverse sources. The process of genomic DNA isolation and purification has evolved considerably within the last decade. The new demands of high throughput facilities have resulted in the development of new technologies for easier and faster DNA processing than ever before. The classic chemical methods for DNA extraction generally is toxic, time consuming, multi step and utilizes organic solvent extraction, alcohol precipitation as well as centrifugation. In the context of bioseparation and purification, nanoparticals technology has become an increasingly popular tool for the separation of biomolecules (e.g. DNA, RNA and proteins) (Bandyopadhyay *et al*., 2011).

Polymerase chain reaction (PCR) employs an *in vitro* DNA replication system by the simultaneous primer extension of complementary strands of DNA, and has become a widely used technique in molecular biology since its invention. The PCR process, which requires very little amount of nucleic acid; can achieve higher detection sensitivity and larger amplification of specific sequences in less time than conventional

methods. Nanomaterials have received considerable attention and nanomaterials-based PCR is a new area in nanobiology that combines artificial nanomaterials and biomolecules, for building and mimicking the DNA replication machinery *in vivo*. Various kinds of nanomaterials have been beneficial to PCR reactions. As the first nanomaterials applied in PCR reaction, single walled carbon nanotubes (in concentrations of < 3 μg/μl) were found to be capable of increasing the amount of PCR product. Gold nanoparticles used to effectively avoid nonspecific PCR amplification, Nanoparticles used to optimized PCR and increase the efficiency by adding such a nanoparticle into the PCR reagent. Since then the combination of some nanoparticals and PCR reactants has been very active, and the number of PCR-improving nanomaterials, which are available, highly stable and well water-soluble, has been in rapid rise, attracting tremendous academic and industrial attentions in nanomaterials-based PCR (Dun *et al*., 2011).

Bacterial conjugation is the transfer of genetic material between bacterial cells by direct cell-to-cell contact or by a bridge-like connection between two cells. Conjugation is a mechanism of horizontal gene transfer as are transformation and transduction although these two other mechanisms do not involve cell-to-cell contact. Bacterial conjugation is often regarded as the bacterial equivalent of sexual reproduction or mating since it involves the exchange of genetic materials. Most widely used methods for DNA transformation of microorganism are chemical transformation and electroporation.

However, both methods require preparation of competent cells and recovery, which are time-consuming and tedious. Furthermore, existing electroporation technology is limited in its ability to treat large quantities of cells and DNA. Additionally, the application of an expensive

electroporation apparatus and a specialized power supply can lead to irreversible electroporation and, consequently, cell lysis. In some routine experiments, high efficiency is as important as time and convenience. Thus, Yoshida and colleagues published many exciting papers describing a novel transformation method based on mineral nanofibers (Yoshida, 2007; Haidang, 2010).

The rapidly growing field of molecular biology and biotechnology has tremendous need for quick, simple robust techniques in genetic testing, so the techniques that need days or week made in a few hours or minutes. Nowadays nanomaterials have received considerable attention because of their promising applications.

According to great importance of nanoparticle applications, and due to limited studies of nanoparticle applications in molecular biology, **this study aimed to** investigate the effect of using nanoparticles with some molecular techniques in order to improve them.

To achieve this aim, Gram negative bacteria and two nanoparticles (ZnO and TiO_2) were selected. Genomic DNA was isolated from bacteria using a number of standard methods and these methods were modified with using different concentrations of ZnO and TiO_2. Genomic DNA was subjected to amplification by polymerase chain reaction, and PCR solutions, reaction conditions and programming were modified with using these nanoparticles. Also transformation and conjugation experiments were performed in presence of ZnO and TiO_2 nanoparticles.

1.2 Literature Review

1.2.1 History of nanotechnology in medicine:

Nanotechnology refers to molecular devices smaller than 1 μm on the nano scale. One nanometer (nm) is one billionth or 10^{-9} of a meter. The field was originally inspired by a talk there's plenty of room at the bottom, by Richard "Feynman in 1959" at the American Physical Society. "Feynman" suggested a number of concepts, including print font size, which would permit the Encyclopedia Britannica to fit on the head of a pin; a feat since accomplished. The broader concept was that because of their small size, nanomaterials have unique qualities that are not found in the same materials at larger sizes. Principles developed from nanotechnology research are being used to develop everything from the next generation of computer chips to fluid-handling devices that will markedly miniaturize current devices. Importantly, the field of Nano Electro Mechanical Systems (NEMS) will be important in implantable devices for a range of biological systems from stress sensors in aneurysms to neural implants. Soon, after the development of mechanical and electrical approaches in nanotechnology, biologists began to explore direct applications using this technology (Dun *et al.*, 2011). Biological approaches and novel applications for molecular nanotechnology was the first scientific conference held on the topic in 1996. The initial focus was small robots that create billions of tiny factories small enough to work within a single cell, but this proved to be more dream than scientific endeavor. However, it became clear that biological systems are organized at nanoscale dimensions and synthetic nanomaterials correlated in size with biological structures such as proteins, glycolipids and DNA. Unique interactions between synthetic nanomaterials and more complex

biological systems were also observed, most likely due to their size. These ranged from good (deliver of materials across the gut) to potentially dangerous (ability of nanoparticles to enter the brain). It was also discovered that the detrimental activities of some types of environmental materials, such as diesel exhaust, was due to their nanoscale dimensions. Building on these discoveries, scientists using nanostructures for biological applications based on their unique capabilities to traverse and interact with similarly sized biological materials. Nanotechnology remains at the far front of medicine and biological technologies from a research perspective (Manoj, 2011).

1.2.2 Properties of Nanoparticles:

Material properties depend on structure and composition and can typically be engineered or modified by changing the relative influence of interfacial or interphase properties and the macroscopic bulk properties through the characteristic size or dimension of components and domains. This approach had already emerged centuries ago with steel alloys and has been so powerful that many engineering materials are composites with micro to nanoscale domain sizes (Li and Liu, 2014).

1.2.3 Nanoparticles: physical and chemical properties:

Depending on the physical or chemical character of each domain, there is a complex interrelation between the structure and the composition of the material, which may relate to the bulk and surface properties of each ingredient and newly emerging properties localized at the interface. There are many properties that distinguish the nanoscale including the size and shape that represent optical properties of nanostructure metal particles,

which depends heavily not only on their dimensions but also on their shape. (Jain and Huang, 2008).

The principal parameters of nanoparticles are their shape (including aspect ratios where appropriate), size and the morphological sub-structure of the substance. Nanoparticles are presented as an aerosol (mostly solid or liquid phase in air), a suspension (mostly solid in liquids) or an emulsion (two liquid phases) in the presence of chemical agents (surfactants), the surface and interfacial properties may be modified. Indirectly such agents can stabilize against coagulation or aggregation by conserving particle charge and by modifying the outmost layer of the particle (Liufu *et al.*, 2005). At the nanoparticle-liquid interface, polyelectrolytes have been utilized to modify surface properties and the interactions between particles and their environment. They have been used in a wide range of technologies, including adhesion, lubrication, stabilization and controlled flocculation of colloidal dispersions) (Warren, 2007).

Both physical and chemical properties are derived from atomic and molecular origin in a complex way. For example, the electronic and optical properties and the chemical reactivity of small clusters are completely different from the better known property of each component in the bulk or at extended surfaces. Complex quantum mechanical models are required to predict the evolution of such properties with particle size and typically very well defined conditions are needed to compare experiments and theoretical predictions (Singh *et al.*, 2010)**.**

The nanoparticle properties depend on phase composition, microstruture, crystallinity and chemical composition, which can be modified by preparation techniques, thermal treatments and metal ion

doping. Nanoparticles crystallize into three natural phases: brookite (orthorhombic), anatase (tetragonal) and rutile (tetragonal). All have the same chemistry, but they have different structures. The brookite and anatase crystalline phases, which are stable at low temperatures, transform into rutile when the sample is calcite at high temperature. It has been demonstrated that some properties of nanoparticles are very sensitive to its structure. Since the anatase phase is chemically and optically active, it is suitable for catalysts and supports (Zhang *et al.*, 2011).

1.2.4 Classification of nanoparticles

Nanoparticles can be broadly grouped into two: namely organic and inorganic nanoparticles. Organic nanoparticles may include carbon nanoparticles (fullerenes) while some of the inorganic nanoparticles may include magnetic nanoparticles, noble metal nanoparticles (like gold and

silver) and semiconductor nanoparticles (like titanium dioxide and zinc oxide (figure 1-1) (Liu *et al*., 2011).

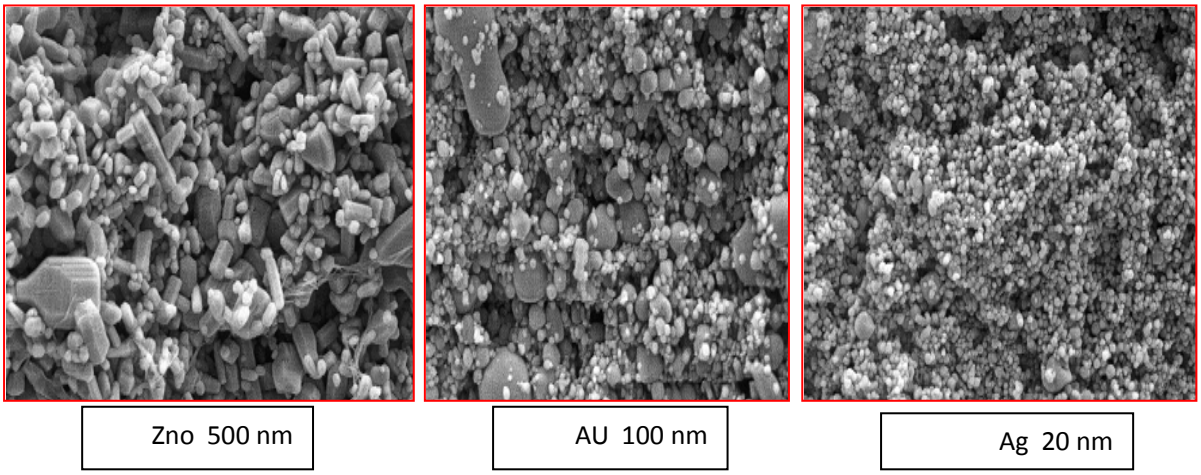


Figure (1-1) Noble metal and semiconductor nanoparticles (Brown *et al*., 2000).

There is a growing interest in inorganic nanoparticles as they provide superior material properties with functional versatility. Due to their size features and advantages over available chemical imaging drugs agents and drugs, inorganic nanoparticles have been examined as potential tools for medical imaging as well as for treating diseases. Inorganic nanomaterials have been widely used for cellular delivery due to their versatile features like wide availability, rich functionality, good biocompatibility and capability of targeted drug delivery and controlled release of drugs (Xu *et al*., 2006). For example, mesoporous silica when combined with molecular machines proves to be excellent imaging and drug releasing systems. Gold nanoparticles have been used extensively in imaging, as drug carriers and in thermo therapy of biological targets (Cheon and Horace, 2009). Inorganic nanoparticles (such as metallic and semiconductor nanoparticles) exhibit intrinsic optical properties which may enhance the transparency of polymer-particle composites. For such reasons, inorganic nanoparticles have found special interest in studies devoted to optical properties in composites. For instance, size dependant color of gold nanoparticles has been used to color glass for centuries (Caseri, 2009).

1.2.5 Strategies used to synthesize nanoparticles:

Previously nanoparticles were produced only by physical and chemical methods. Some of the commonly used physical and chemical methods are ion sputtering, solvothermal synthesis, reduction and sol gel technique. Basically there are two approaches for nanoparticle synthesis namely the bottom up approach and the top down approach. In the top down approach, it is about formulating nanoparticles using larger ones to direct their assembly. The bottom up approach is a process that builds towards

larger and more complex systems by starting at the molecular level and maintaining precise control of molecular structure (Guo and Wang, 2007).

❖ Top-down method: top-down method refers to a set of fabrication technologies which fabricate by removing certain parts from a bulk material substrate. The removing methods can be mechanical, chemical, electrochemical etc., depending on the material of the base substrate and requirement of the feature sizes (figure 1-2).

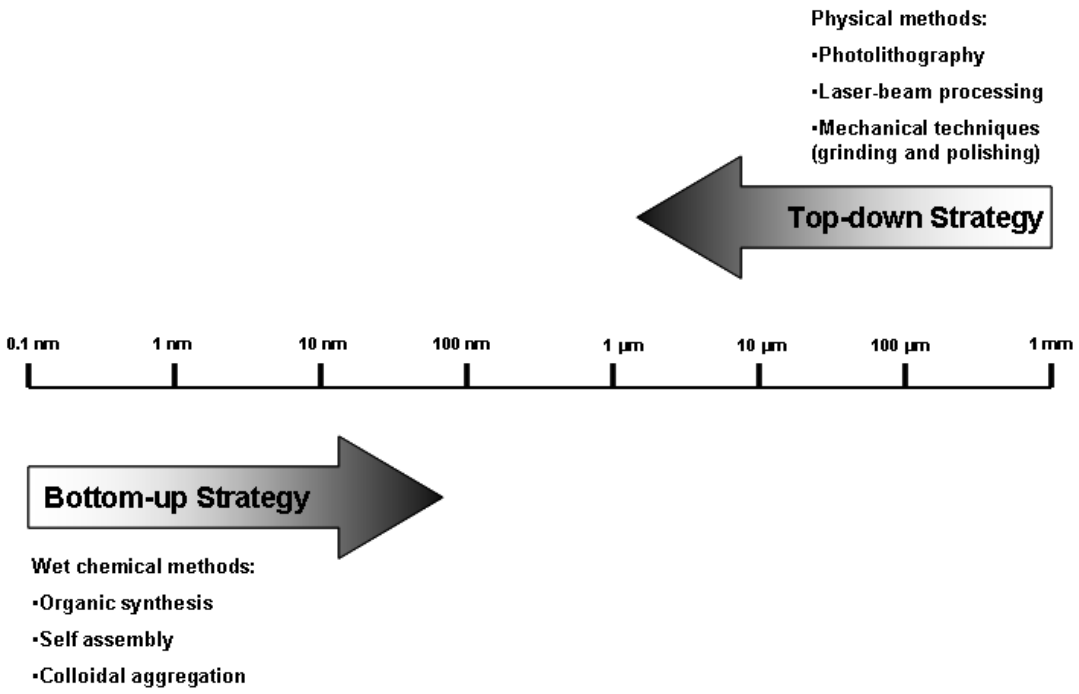


Figure (1-2) Strategies of synthesize nanoparticles (Ju-Nam *et al.*, 2008).

The formed structures usually share the same material with the base substrate. There are a couple of manufacturing technologies in the conventional scale which can be categorized top-down (Gao, 2004; Ju-Nam *et al.*, 2008). Milling is a representative example. In the milling

process, a certain material is selectively removed from the substrate, usually a metal sheet, forming a cavity with certain geometries. The dimensions of the cavity depend on the travel path of the mill, which can be precisely controlled with the help of computer assisted numerical systems. The milling technique, along with similar methods such as drilling and grinding, is the most widely used technique in conventional manufacturing industry. People have attempted to extend top-down method into nanometer domain and supplemented the mechanical removing methods with chemical and electrochemical methods (figure1-2).

❖ Bottom-up method: as the opposite of top-down fabrication technologies, bottom-up methods refer to a set of technologies which fabricate by stacking materials on top of a base substrate (Tolaymat *et al*., 2010). These methods are similar in principle to welding and riveting at the conventional scale, in which a different type of material is attached to the base component by melted solder or physical fitting. In welding and riveting, attention is mainly paid to the strength of the contact area in order to maintain the construct as a reliable component for high load application. Similarly, in bottom-up nanofabrication, the adhesion of the surface layer to the base substrate is also an important concern. There is extensive research on the surfactants to enhance adherence and avoid cracks during the subsequent processing. Research has also focused on autonomous patterning of the surface layer into nanometer scale features since, manipulation of nanoscale components is not ever an easy task as compared to that at the conventional scale (figure 1-2). (Rosa *et al*., 2013).

Methods for nanoparticle synthesis:

Some of the commonly used physical and chemical and biologi methods include: biometrics synthesis of nanoparticles:

- Chemical reduction, which is the reduction of an ionic salt in an appropriate medium in the presence of surfactant using reducing agents (Wang *et al*., 2005).
- Solvothermal synthesis, which is a versatile low temperature route in which polar solvents under pressure and at temperatures above their boiling points are used. Under solvothermal conditions, the solubility of reactants increases significantly, enabling reaction to take place at lower temperature (Gersten, 2013).
- Sol-gel technique, which is a wet chemical technique used for the fabrication of metal oxides from a chemical solution which acts as a precursor for integrated network (gel) of discrete particles or polymers. The precursor sol can be either deposited on the substrate to form a film, cast into a suitable container with desired shape or used to synthesize powders (Epifani *et al*., 2000; Hayle and Gonfa, 2014).
- Inert gas condensation, where different metals are evaporated in separate crucibles inside an ultra-high vacuum chamber filled with helium or argon gas at typical pressure of few 100 Pascal's. As a result of inter atomic collisions with gas atoms in chamber, the evaporated metal atoms lose their kinetic energy and condense in the form of small crystals which accumulate on liquid nitrogen filled cold finger (Gracia *et al*., 2010).

- Green Synthesis: They have investigated in order to find an eco-friendly technique for production of well-characterized nanoparticles.

Among these organisms plants seem to be the best candidates and they are suitable for large-scale biosynthesis of nanoparticles. Nanoparticles produced by plants are more stable and the rate of synthesis is faster than in the case of microorganisms. Moreover, the nanoparticles are more various in shape and size in comparison with those produced by other organisms (Siavash, 2011).

1.2.6 Types of Nanoparticles

The dimension of material important in nanoscience and nanotechnology is typically on the 1 to 100 nm scale (nanoscale). The properties of materials change as their size approaches the nanoscale. Further, the percentage of atoms at the surface of a material becomes more significant (Eustis, 2006). Bulk materials possess relatively constant physical properties regardless of their size, but at the nanoscale this is often not the case. As the material becomes smaller the percentage of atoms at the surface increases relative to the total number of atoms of the material bulk. This can lead to unexpected properties of nanoparticles which are partly due to the surface of the material dominating over the bulk properties. At this scale, the surface-to-volume ratios of materials become large and their electronic energy states become discrete, leading to unique electronic, optical, magnetic, and mechanical properties of the nanomaterials (Gurin and Alexeenko, 2010). In general, as the size of inorganic and organic materials decreases towards the nanoscale, their optical and electronic properties largely varies from the bulk material at the atomic/molecular levels and is size and shape dependent. The various size dependent properties that can be observed are quantum confinement in semi-conductor particles, surface plasmon resonance in noble metal particles and super paramagnetism in magnetic materials. Thus, the

crystallographic surface structure and the large surface to volume ratio make the nanoparticles exhibit remarkable properties. Moreover, the increased catalytic activity due to morphologies with highly active surfaces and the tailoring of its synthesis as per the requirement makes the nanoparticles an attractive tool to solve various technological problems (Jamieson *et al*., 2007).

- **Liposomes**

Liposomes are concentric bilayered vesicles in which an aqueous volume is entirely enclosed by a membranous lipid bilayer mainly composed of natural or synthetic phospholipids. Liposomes are characterized in terms of size, surface charge and number of bilayers. It exhibits number of advantages in terms of amphiphilic character, biocompatibility, and ease of surface modification rendering it a suitable candidate delivery system for biotechnology and drugs. Liposomes have been used successfully in the field of biology, biochemistry and medicine since its origin (Manju *et al*., 2006).

- **Nanocrystals and nanosuspension**

Nanocrystals are aggregates of around hundreds or thousands of molecules that combine in a crystalline form, composed of pure drug with only a thin coating comprised of surfactant or combination of surfactants. It is important in drug delivery because a minimum quantity of surfactants needs to be added in nanocrystals for steric and electrostatic surface stabilization. Moreover, administration of high drug levels with depot release can be achieved if dissolution is sufficiently slow. As pure drug is used and no carrier is needed, eliminating potential toxicity issues associated with the carrier molecule (Patel, 2011).

- **Solid lipid nanoparticles**

Solid lipid nanoparticles (SLN) were developed at the beginning of the 1990s as an alternative carrier system to emulsions, liposomes and polymeric nanoparticles as a colloidal carrier system for controlled drug delivery. It consists of a solid lipid matrix, where the drug is normally incorporated, with an average diameter below 1 µm. To avoid aggregation and to stabilize the dispersion (Patil *et al.*, 2013).

- **Polymeric nanoparticles**

In comparison to SLN or nanosuspensions, polymeric nanoparticles (PNPs) consist of a biodegradable polymer. Biocompatibility is an essential feature for potential application as tissue engineering, drug and gene delivery and new vaccination strategies (Mandal *et al.*, 2012).

- **Dendrimers**

Dendrimers, a unique class of polymers, are highly branched macromolecules whose size and shape can be precisely controlled. Dendrimers are fabricated from monomers using either convergent or divergent step growth polymerization. There are two representations of polyamidoamine- based dendrimers. The welled fined structure, monodispersity of size, surface functionalization capability, and stability are properties of dendrimers that make them attractive drug carrier candidates (Malik *et al*., 2012).

- **Silcon-based structures**

Silicon-based structures can be fabricated by photolithography, etching, and deposition techniques commonly used in the manufacture of semiconductors and microelectromechanical systems (MEMS). The most commonly investigated silicon-based materials for drug delivery are

porous silicon and silica, or silicon dioxide. Architectures include calcified nanopores, platinum-containing nanopores, porous nanoparticles, and nanoneedles examples of therapies being investigated for use with silicon-based delivery systems include porous silicon embedded with platinum as an antitumor agent, silicon nanopores for antibody delivery, and porous silica nanoparticles containing antibiotics, enzymes, and DNA (Alexander, 2013).

- **Carbon structures**

Two nanostructures that have received much attention in recent years are hollow, carbon-based, cage-like architectures: nanotubes and fullerenes, also known as buck balls. Single-wall nanotubes (SWNTs), multiwall nanotubes (MWNTs), and C60 fullerenes are common configurations. The size, geometry, and surface characteristics of these structures make them appealing for drug carrier usage. SWNTs and C60 fullerenes have diameters on the order of 1nm, about half the diameter of the average DNA helix. Other simulations have involved the transport of DNA through CNTs, indicating potential use as a gene delivery tool. For example, temperature-stabilized hydrogels for drug delivery applications incorporate CNTs. Fullerenes have also shown drug targeting capability (figure 1-3) (Abhilash, 2010).

- **Metal structures**

Hollow metal nanoshells are being investigated for drug delivery applications. Typical fabrication methods involve templeting of the thin metal shell around a core material such as a silica nanoparticle. Typical metals include gold, silver, platinum, and palladium (Gareth, 2005).

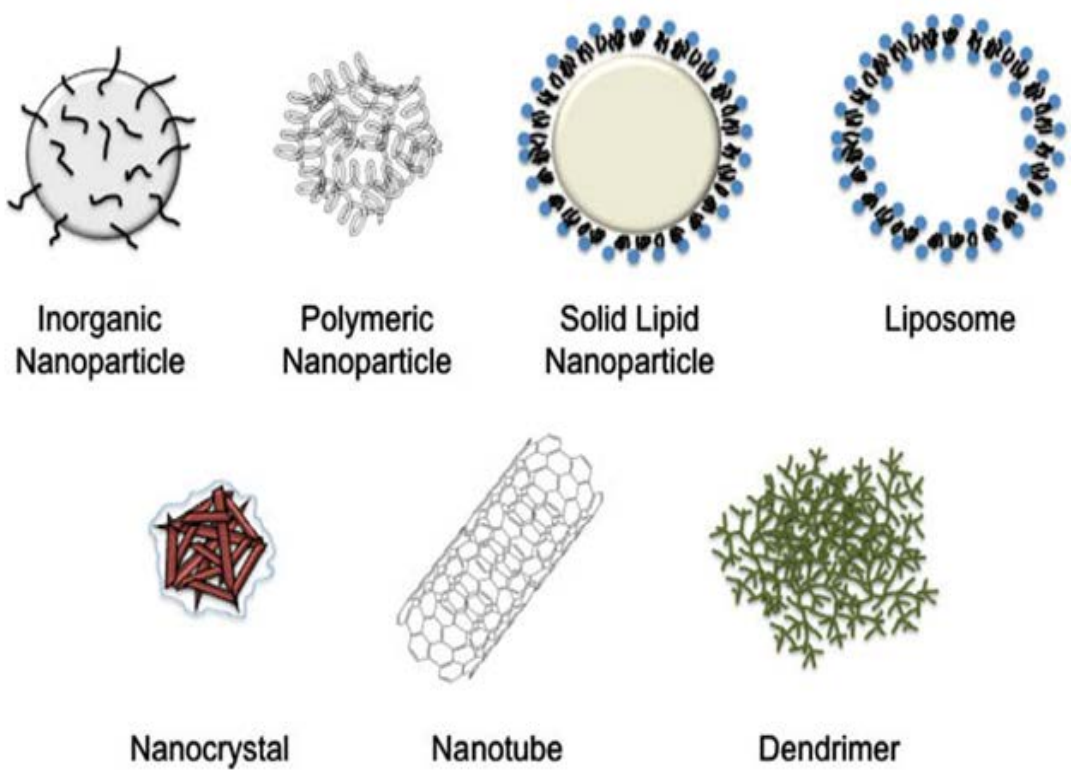


Figure (1-3) Types of nanoparticals (Abhilash, 2010).

- **Zinc oxide nanoparticles (ZnO):**

Zinc oxide nanoparticles explored for applications range from sunscreens, to advanced textiles, to self charging electronic device.

Among the various metal oxides studied for their antibacterial activity, zinc oxide nanoparticles have been found to be highly toxic. Moreover, their stability under harsh processing conditions and low toxicity combined with the potent antimicrobial properties favours their application as antimicrobials (Stoimenov, 2002). Many studies have shown that some NPs made of metal oxides, such as ZnO NP, have selective toxicity to bacteria and only exhibit minimal effect on human cells, which recommend their prospective uses in agricultural and food industries (Brayner *et al*., 2006; Zhang *et al*., 2007). It was also shown that; ZnO nanoparticles (12 nm) inhibited the growth of *E. coli* by

disintegrating the cell membrane and increasing the membrane permeability (Jin *et al*., 2009). The above findings suggest that ZnO nanoparticles can find applications in food systems and can be used to inhibit growth of pathogenic bacteria. Application of ZnO nanoparticles for DNA extraction is so rare, but from these rare experiments it was found that ZnO can be used as a solid face absorbent for plasmid DNA purification, as improving reagent for PCR and as novel carriers for mammalians cell transfection (Leng *et al*., 2006).

- **Titanium dioxide nanoparticles (TiO_2):**

Titania (TiO_2) has been widely studied because of its many useful optical, electrical and photocatalytic properties, which depend on the crystalline structure of TiO_2.Titanium dioxide (TiO_2) particles with diameter larger than 100 nm are considered biologically inert in both humans and animals. Based on this understanding, titanium dioxide nanoparticles have been widely used in many products, such as white pigment, food colorant, sunscreens and cosmetic creams (Gurr *et al*., 2005). New research is exploring the potential use of nanostructured titanium dioxide photocatalyst materials for sterilizing equipment of environmental microorganisms in the health care facility. Coatings of nanoparticles are widely used for modifying fabrics to create stain and wrinkle free properties. In addition, one can find clothes with built-in sunscreen and moisture management technology. Fabric containing bamboo-charcoal nanoparticles claims antibacterial antifungal properties. The inhibitory activity of TiO_2 is due to the photocatalytic generation of strong oxidizing power when illuminated with UV light at wavelength of less than 385 nm, the use of TiO_2 photocatalysts as alternative means of self-disinfecting contaminated surfaces by further development may provide potent disinfecting solutions for prevention of biofilm formation.

It has been suggested that nanostructured TiO_2 on UV irradiation can be used as an effective way to reduce the disinfection time, eliminating pathogenic microorganisms in food contact surfaces and enhance food safety (Asthanal *et al*., 2010).

1.2.7 Application of nanoparticles

Nanoscience represents one of the most rapidly growing research areas, allowing the manipulation of material at the nanoscal and allowing the controlled fabrication of such systems and devices. Engineered nanoparticles offer potential applications in many areas beneficial for human kind, including sensors, medical imaging, drug delivery system, cosmetics, and many others (Baker and Pradhan, 2004).

It is evident that the metal based nanoparticles constitute an effective antimicrobial agent against common pathogenic microorganisms. Therefore, some of the nanoparticles such as silver, titanium dioxide and zinc oxide are receiving considerable attention as antimicrobials and additives in consumer, health-related and industrial products. Nanoparticles of titanium dioxide are used in cosmetics, filters that exhibit strong germicidal properties and remove odors, and in conjunction with silver as an antimicrobial agent. Moreover, due to the photocatalytic activity, it has been used in waste water treatment. Titanium dioxide nanoparticles are finding wide application as a self-cleaning and self-disinfecting material for surface coatings in many applications and in food industries for disinfecting equipments (Wist *et al*., 2004). Zinc oxide (ZnO) and copper oxide nanomaterials due to their antimicrobial property are being incorporated into a variety of medical and skin coatings. ZnO nanoparticles are used in the wallpapers in hospitals as antimicrobials. ZnO powder is an active ingredient for dermatological applications in

creams, lotions and ointments on account of its antibacterial properties (Martinez *et al*., 2003).

1.2.7.1 Molecular genetics:

DNA extraction and gene amplification by using PCR technique:

The rapidly growing field of molecular biology and bio- technology has a tremendous need for quick, simple, robust and high-throughput procedures for extraction of DNA from diverse sources. The process of genomic DNA isolation and purification has evolved considerably within the last decade. The new demands of high-throughput facilities have resulted in the development of new technologies for easier and faster DNA processing than ever before. The classic chemical methods for DNA extraction generally is toxic, time-consuming, multi-step and utilizes organic solvent extraction, alcohol precipitation as well as centrifugation. In the context of bioseparation and purification, nanoparticles technology has become an increasingly popular tool for the separation of biomolecules (e.g. DNA, RNA and proteins) (Bandyopadhyay *et al*., 2011).

Polymerase chain reaction (PCR) employs an *in vitro* DNA replication system by the simultaneous primer extension of complementary strands of DNA and has become a widely used technique in molecular biology since its invention. Compared with traditional methods of cloning a DNA sequence into a vector and replicating it in a living cell, amplification of DNA sequences by PCR requires only several hours rather than several days or weeks. Unlike most biochemical analyses (including nucleic acid detection with radioisotopes) that requires significant amounts of biological material, the PCR process, which requires very little, can achieve higher detection sensitivity and larger amplification of specific

sequences in less time than conventional methods (Abdul Khaliq *et al* ., 2011) Therefore, the PCR technique is extremely useful in basic research and commercial uses as well, such as genetic identity testing, forensics, industrial quality control and *in vitro* diagnostics. However, PCR amplification, as the replication machinery *in vitro*, is not completely reconstituted *in vitro*, but rather patched together from a few key components, hence the necessity of improving the key components, in some cases, for their application *in vitro*. For example, heat-stable DNA polymerase (Jopbling and Gill, 2004), instead of polymerase *in vivo*, simplified and improved the operation of PCR. Nanomaterials have received considerable attention because of their promising applications in biology and medicine. However, nanomaterials-based PCR is a new area in nanobiology that combines artificial nanomaterials and biomolecules, for building and mimicking the DNA replication machinery *in vivo*. Nanoparticle biomolecule interactions are widely used in drug and gene delivery, biological sensing, and imaging of living cells and tissues. Nanometer sized materials, with unique physical and chemical properties, have been widely used for years. Macro- biomolecules, in nanometers in size, too, possess functionalities that enable recognition and modulation. Therefore, the nanoparticles, of large surface-area/volume ratio, may interact with functional groups in the biomolecule surface (Banerjee *et al*., 2010).

Various kinds of nanomaterials have been beneficial to PCR reactions. As the first nanomaterials applied in PCR reaction, single walled carbon nanotubes were found in 2004 to be capable of increasing the amount of PCR product (Li *et al*., 2005). They optimized their PCR using AuNPs and increased the PCR efficiency by adding AuNPs into the PCR reagent. Since then the combination of AuNPs and PCR reactants has been very

active, and the number of PCR-improving nanomaterials, which are available, highly stable and well water soluble, has been in rapid rise, attracting tremendous academic and industrial attentions in nanomaterials-based PCR. Emphasis is given on metal nanoparticles (NPs), metal oxide NPs, and non- metal nanomaterials including nanometer-sized polymers, which are able to optimize conventional PCR amplification with their unique chemical, physical or optical properties (Dun *et al*., 2011).

1.2.7.2 Transformation and Conjugation:

In molecular biology, transformation is the genetic alteration of a cell resulting from the direct uptake and incorporation of exogenous genetic material (exogenous DNA) from its surroundings and taken up through the cell membrane. Transformation occurs naturally in some species of bacteria, but it can also be affected by artificial means in other cells. For transformation to happen, bacteria must be in a state of competence, which might occur as a time-limited response to environmental conditions such as starvation and cell density (Albert, 2002).

Transformation was first demonstrated in 1928 by British bacteriologist Frederick Griffith. Griffith discovered that a harmless strain of *Streptococcus pneumoniae* could be made virulent after being exposed to heat-killed virulent strains. Griffith hypothesized that some "transforming principle" from the heat-killed strain was responsible for making the harmless strain virulent. In 1944 this "transforming principle" was identified as being genetic by Oswald Avery, Colin MacLeod, and Maclyn McCarty. They isolated DNA from a virulent strain of *S. pneumoniae* and using just this DNA was able to make a harmless strain virulent. They called this uptake and incorporation of DNA by bacteria

"transformation". However, in 1970, Morton Mandel and Akiko Higa showed that *E. coli* may be induced to take up DNA from bacteriophage λ without the use of helper phage after treatment with calcium chloride solution. The method of transformation by Mandel and Higa was later improved upon by Douglas Hanahan. The discovery of artificially induced competence in *E. coli* created an efficient and convenient procedure for transforming bacteria which allows for simpler molecular cloning methods in biotechnology and research, and it is a routinely used laboratory procedure (Lederberg, 1994).

Transformation using electroporation was developed in the late 1980s, increasing the efficiency of *in-vitro* transformation and increasing the number of bacterial strains that could be transformed. Many methods are used for transformation including natural transformation which is a bacterial adaptation for DNA transfer that depends on the expression of numerous bacterial genes whose products appear to be designed to carry out this process. In general, transformation is a complex, energy requiring developmental process (Johnsborg *et al.*, 2007).

Natural competence about 1% of bacterial species are capable of naturally taking up DNA under laboratory conditions; more may be able to take it up in their natural environments. DNA material can be transferred between different strains of bacteria, in a process that is called horizontal gene transfer. Some species upon cell death release their DNA to be taken up by other cells; however transformation works best with DNA from closely related species (Donahue, 1998).

Artificial competence can be induced in laboratory procedures that involve making the cell passively permeable to DNA by exposing it to conditions that do not normally occur in nature. Typically the cells are incubated in a solution containing cations (often calcium chloride) under

cold conditions, before being exposed to a heat pulse (heat shock). It has been found that growth of Gram negative bacteria in Mg reduces the number of protein to lipopolysaccharide bonds by increasing the ratio of ionic to covalent bonds, which increases membrane fluidity, facilitating transformation. Exposing the cells to cation in cold condition may change or weaken the cell surface structure of the cells making it more permeable to DNA. The heat-pulse is thought to create a thermal imbalance on either side of the cell membrane, which forces the DNA to enter the cells through either cell pores or the damaged cell wall. Electroporation is another method of promoting competence. In this method the cells are briefly shocked with an electric field of 10-20 kV/cm which is thought to create holes in the cell membrane through which the plasmid DNA may enter. After the electric shock the holes are rapidly closed by the cell's membrane-repair mechanisms (Srivastava, 2013).

Most widely used methods for DNA transformation of eukaryotes are chemical transformation and electroporation. However, both methods require preparation of competent cells and recovery, which are time-consuming and tedious. Furthermore, existing electroporation technology is limited in its ability to treat large quantities of cells and DNA. Additionally, the application of an expensive electroporation apparatus and a specialized power supply can lead to irreversible electroporation and, consequently, cell lysis. In some routine experiments, high efficiency is as important as time and convenience. Thus, many exciting papers have been published to describe a novel transformation method based on mineral nanofibers. In any way, based on nanomaterials, the transformation offer several advantages over the chemical method and electroporation: (1) the experiment can be performed conveniently at any time. For instance, DNA transformation will occur just by shaking when

the nanosolution is mixed with heterogeneous DNA and bacteria in a microtube; (2) small amount of cells can be transformed by plasmids easily. For example, a single colony can be transformed by plasmids directly even when the colony has been stored on a plate at 4 °C for more than one month; (3) the experiment for the reception of some pathogens to various plasmids can be performed easily based on nanomaterials (Yoshida, 2007).

Bacterial conjugation is the transfer of genetic material between bacterial cells by direct cell-to-cell contact or by a bridge-like connection between two cells. Discovered in 1946 by Joshua Lederberg and Edward Tatum, conjugation is a mechanism of horizontal gene transfer as are transformation and transduction although these two other mechanisms do not involve cell-to-cell contact. Bacterial conjugation is often regarded as the bacterial equivalent of sexual reproduction or mating since it involves the exchange of genetic material. During conjugation the donor cell provides a conjugative or mobilizable genetic element that is most often a plasmid or transposon. Most conjugative plasmids have systems ensuring that the recipient cell does not already contain a similar element (Menintz *et al*., 2005). The genetic information transferred is often beneficial to the recipient. Benefits may include antibiotic resistance, xenobiotic tolerance or the ability to use new metabolites. Other elements, however, may be viewed as bacterial parasites and conjugation as a mechanism evolved by them to allow for their spread (Russi *et al*., 2008).

1.2.7.3 Other applications:

Applications entering widespread use include: fabrics and their treatments, filtration, dental materials, surface disinfectants, diesel and

fuel additives, hazardous chemical neutralizers, automotive components, electronics, scientific instruments, sports equipment, drug delivery systems, and pharmaceutics. The unique properties of nanomaterials encourage the belief that they can be applied in a wide range of fields, from medical applications to environmental sciences. Studies conducted by nanotechnology experts mapping the risks and opportunities of nanotechnology have revealed enormous prospects for progress in both life sciences and information technology. Medical and molecular applications are expected to increase our quality of life through early diagnosis and treatment of diseases, and prosthetics (Roco, 2005).

- **Electronics**

Microelectronics: Many of the microelectronics applications are already at a nanoscale. During the last four decades, the smallest feature of a transistor shrunk from 10 μm down to 30 nm (Thompson, 2006).

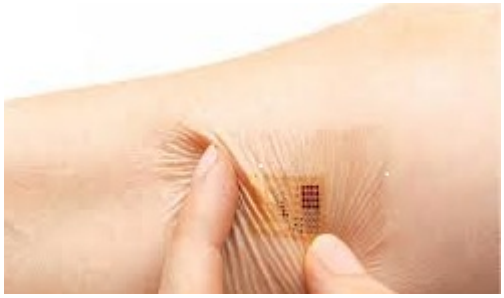


Figure (1-4) Goldnanoparticles in electronics (Thompson, 2006).

- **Imaging**

Scanning microscope imaging, have been used as probe tips for atomic-force microscopy imaging of antibodies, DNA, etc. Nanotubes are ideal probe tips for scanning microscopy due to their small diameter (which maximizes resolution), high aspect ratio, and stiffness (Hafner *et al*., 2001).

❖ Biomedical applications

Nanofiber can be used to regenerate central nervous system cells and possible other organs. Experiments performed on a hamster with severed optic tract demonstrated the regeneration of axonal tissue initiated by peptide nanofibers. Antimicrobial nanopowders and coatings, certain nanopowders, possess antimicrobial properties. When these powders contact cells of *E. coli*, or other bacteria species and viruses, over 90% are killed within a few minutes (Elis-Behnke *et al*., 2006).

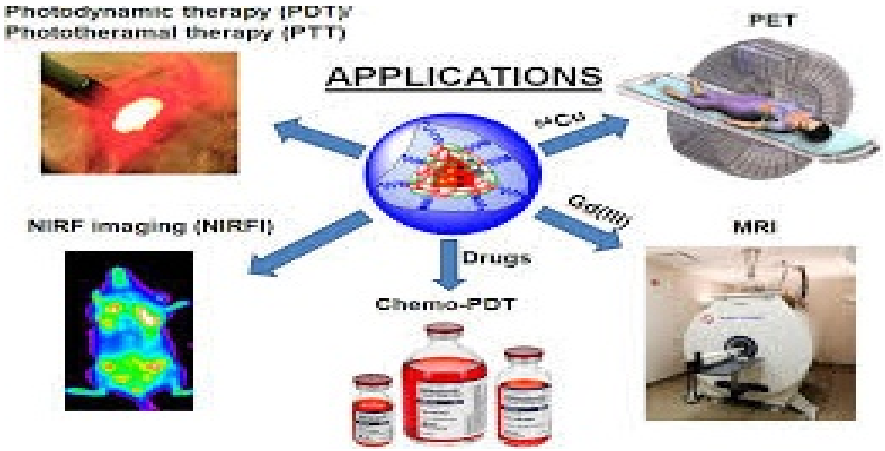


Figure (1-5) Some biomedical application (Elis-Behnke *et al*., 2006).

❖ Bioseparation

Nanotube membranes can act as channels for highly selective transport of molecules and ions between solutions that are present on both side of the membrane. For example, membranes containing nanotubes with inside diameters of molecular dimensions (less than 1 nm) separate small molecules on the basis of molecular size, while nanotubes with larger inside diameters (20–60 nm) can be used to separate proteins (Martine and kohli, 2003).

❖ **Drug delivery**

The ability of nanoparticles to target and penetrate specific organs and cells contributes to their toxicity; however, this ability may be exploited in nanomedicine. Nanospheres composed of biodegradable polymers can incorporate drugs, allowing the timed release of the drug as the polymer degrades (Kneuer *et al.*, 2000).

❖ **Medical imaging**

A variety of techniques called “non-invasive” have been used for more than a quarter of a century in medical imaging. Intracellular imaging is also possible through attachment of quantum dots to selected molecules, which allows intracellular processes to be observed directly. Nanospheres carriers for vaccines are in development. Antigen-coated polystyrene nanospheres, used as vaccine carriers targeting human dendritic cells, have been researched for nasal vaccination (Matsusaki *et al*., 2005). Nanomaterials-based assays are evaluated and more sensitive proteins detection methods. Nucleic acid sequences are currently detected with polymerase chain reaction (PCR) coupled with molecular fluorophore assays. More sensitive methods based on nanomaterials would revolutionize physical treatment of many cancer types and diseases. Differentially functionalized nanotubes are used as smart nanophase extractors, with molecular-recognition capabilities, to remove specific molecules from solutions, (Renehan *et al*., 2005).

❖ **Pollution remediation**

Although research on environmental applications of nanoparticles is a new area, it is growing rapidly. The potential of nanoparticles to react with pollutants in the air, soil, and water and transform them into

harmless compounds is currently being researched. Elimination of pollutants due to their enhanced chemical activity, nanomaterials can be used as catalysts to react with toxic gases in automobile catalytic converters and power generation equipment. This could prevent gaseous environmental pollution arising from burning gasoline and coal. Paints that absorb noxious gases from vehicle exhaust have already been developed (Hogan, 2004). The nanoparticles are able to remove organic chlorine (a carcinogen) from water and soil contaminated with the chlorine-based organic solvents (used in dry cleaners) and convert the solvents to benign hydrocarbons (He and Zhao, 2005).

- **Cosmetics**

Titanium dioxide and zinc oxide become transparent to visible light when formed at the nanoscale, however are able to absorb and reflect UV light, being currently used in sunscreens and in the cosmetic industry (Birhanli *et al.*, 2014).

- **Coatings**

Nanomaterials have been used for very thin coatings for decades, if not centuries. Thin coatings are used in a vast range of applications, including architectural glass, microelectronics, anticounterfeit devices, optoelectronic devices, and catalytically active surfaces. Nanoparticles have already been used in coating textiles such as nylon, to provide antimicrobial characteristics. (Baibarac and Gomez-Romero, 2006).

- **Mechanical engineering**

Cutting tools made of nanocrystalline materials (such as tungsten carbide) are much harder than their conventional due to the fact that the microhardness of nanosized composites is increased compared to that of

microsized composites. Nanospheres of inorganic materials could be used as lubricants, acting as nanosized ball bearings (Fleischer *et al*., 2003).

1.2.8 Toxicity of nanoparticles

It was evident from the some studies that metal nanoparticles due to their unique physico-chemical and biological properties have far reaching industrial and medical applications. But there is a dearth of knowledge about the effect of the prolonged exposures to nanoparticle on human health and environment. The implication of nanoparticles on health and environment needs to be assessed completely before their large-scale production and application in various fields (Finney and Halloran, 2003; Sharma *et al*., 2009). Studies conducted on the NP-induced toxicity have revealed that the metal-based nanoparticles can affect the biological behavior at the organ, tissue, cellular, subcellular, and protein levels. The size of the nanoparticles is small and these can easily access the skin, lungs, and brain and cause adverse effects (Jevgenij *et al*., 2014; Huster *et al*., 2007). For example, exposure of metal based nanoparticles to human lung epithelical cells leads to the generation of reactive oxygen species and result in oxidative stress and cellular damage. The toxicity of nanoparticles can be assessed by a number of *in vitro* and *in vivo* studies. For example, the toxic effects of nanoparticles can be carried out using zebrafish as a model due to its fast development and transparent body structure. Cell culture based assays are used as a pre-screening tool to understand the biological effects of nanoparticles. However, along with the *in vitro* assays it is necessary to confirm the *in vivo* biological activities of nanoparticles in animal models to study the suitability of their application (Asharani *et al*., 2008; Shaw *et al*., 2008). It is evident that metal based nanoparticles due to their biological and physiochemical properties are promising as antimicrobials and therapeutic agents. They

can be used to address a number of challenges in the field of nano medicine. But it must be remembered that they can also possibly cause adverse biological effects at the cellular and subcellular levels. Therefore, after the cytotoxicity and clinical studies the nanoparticles can find immense application as antimicrobials in the consumer and industrial products (Schrand *et al*., 2010)

Many sunscreens use nanoparticle titanium dioxide (along with nanoparticle zinc oxide) which, despite reports of potential health risks, is not actually absorbed through the skin. Other effects of titanium dioxide nanoparticles on human health are not well understood Nevertheless, allergy to topical application has been confirmed (Shaw *et al*., 2010) Titanium dioxide dust, when inhaled, has been classified by the International Agency for Research on Cancer (IARC) as an IARC Group 2B carcinogen, meaning it is possibly carcinogenic to humans. The findings of the IARC are based on the discovery that high concentrations of pigment-grade (powdered) and ultrafine titanium dioxide dust caused respiratory tract cancer in rats exposed by inhalation and intratracheal instillation. (Serpone and Kutal,1993).

The safety of the use of nanoparticle sized titanium dioxide, which can penetrate the body and reach internal organs, has been criticized. Titanium dioxide nanoparticles cause inflammatory response and genetic damage in mice. The mechanism by which TiO_2 may cause cancer is unclear. Molecular research suggests that cell cytotoxicity due to TiO_2 results from the interaction between TiO_2 nanoparticles and the lysosomal compartment, independently of the known apoptotic signalling pathways (Zhu *et al.*, 2012).

The body of research regarding the carcinogenicity of different particle sizes of titanium dioxide has led the US National Institute for Occupational Safety and Health to recommend two separate exposure limits. NIOSH recommends that fine TiO_2 particles be set at an exposure limit of 2.4 mg/m^3, while ultrafine TiO_2 be set at an exposure limit of 0.3 mg/m^3, as time-weighted average concentrations up to 10 hours a day for a 40 hour work week (NIOSH, 2011).

Chapter two

Materials And Methods

2. Materials and Methods

2.1 Materials

2.1.1 Apparatus and equipments

The following apparatus and equipments were used in this study:

Equipments	Company/ Country
Autoclave	Express/Germany
Balance	Sartorius/ Germany
Centrifuge	Hermile Z200A /Germany
Compound light microscope	Olympus/ Japan
Cooling centrifuge	Hermile/ Germany
Distillator	GFL/ Germany
Electrophoresis Unit	Bio Rad/ USA
Eppendorf bench centrifuge	Netherler and Hinz (Germany)
Gel Imaging System	Bio Rad/ USA
Hot plate with magnetic stirrer	Gallen Kamp / England
Incubator	Sanyo / Japan
Laminar air flow hood	Sanyo/ Japan
Micropipettes	Eppendroff / Germany
Microwive	Kenwood / China
Millipore filter unit	Millipore corp / USA
NanoDrop spectrophotometer	Biometra / England
Oven	Gallenkamp / England
pH-meter	Martini/ Germany
Refrigerator	Ariston / Japan
Scanning electron microscope	MEIJI/ Japan
Sensitive balance	Denver/ Germany

Shaker incubator	GFL / Germany
Spectrophotometer (UV-VIS)	Aurora instruments Ltd / England
Sonicater	Branson sonifier/USA
Thermo cycle T-5000	Biometra / England
U V Visible-Transiluminator	Vilber Lourmat/ France
Vortex	Stuart/ England
Water bath	Grant/ England

2.1.2 Chemicals and Biological materials

The following chemicals and biological materials were used in this study:

Material	Company/ Country
Agar, Trypton	Biolife/ Italy
Agarose, Ethidium bromide, Lysozyme	Sigma/ USA
Boric acid, Bromo phenol blue	Riedel-Dettaen/ Germany
DNA ladder 100 bp (100-1.500) bp DNA ladder 1 Kb (200-10200) bp	Promega/ USA
Ethanol, Sodium chloride, Sodium hydroxide, potassium acetate, Magnesium sulphate	Sigma /USA
Ethylene diamine tetra-acetic acid, Sodium dodecyl sulphate (SDS), Tris-hydrochloride, Glatial acetic acid, Sodium acetate	Fluka/ Switzerland
Glucose, Sucrose, triton X-100	BDH/England
HCl, Isopropanol, Chloroform, KCl, Glycerol, Sucrose, Calcium chloride magnesium chloride	BDH/England

Peptone, Yeast extract	Himedia/ India

- **Nanoparticles :**

The following nanoparticles were used in this study:

Nanoparticles	Characteristics	Company/ country
Titanium dioxide (TiO_2) colloids nanoparticles	Whitish colloid NPs, spherical in shape under TEM. Purity 99.9% 50-150 nm, Mix rutile and anatase con.33.37 w%, dissolved in water. Mwt 79.90	Sigma/ USA
zinc oxide (ZnO) colloids nanoparticles	Pale yellow colloid Nps , spherical in shape under TEM Purity 99.00% 20-50 nm, anatase, con. 50 w% dissolved in water. Mwt 81.39	Sigma/ USA

2.1.3 Antibiotics

2.1.3.1 Antibiotic discs

The following antibiotic discs were used in this study:

Antibiotics	**Abb.**	**Concentration (µg/ Disc)**	**Company (origin)**
Amikacin	AK	30	Oxoid (England)
Amoxicillin	AX	25	Oxoid
Ampicillin	AM	25	Oxoid
Azithromycin	AZM	15	Oxoid
Cefotaxime	CTX	5	Oxoid
Cephalothin	KF	30	AL - Razzi (Iraq)
Cephradine	CE	30	AL - Razzi
Chloramphenicol	C	10	Oxoid
Ciprofloxacin	CIP	5	Oxoid
Clindamycin	DA	2	AL - Razzi
Doxycycline	DO	30	Oxoid
Novobiocin	NOV	25	Oxoid
Nalidixic acid	NA	30	AL – Razzi
Norfloxacillin	Nor	10	AL – Razzi
Penicillin	P	10\IU	AL – Razzi
Rifampicin	RA	30	Oxoid
Tetracycline	TE	30	Oxoid
Vancomycin	VA	30	AL – Razzi

2.1.3.2 Antibiotic Powders:

Antibiotic	Cod	Company (origin)
Ampicillin	AM	Oxoid (England)
Tetracycline	TE	Oxoid
Cefotaxime	CTX	Oxoid
Rifampicin	RA	Oxoid

2.1.4 Bacterial strains

The following bacterial strains used in this study:

Bacterial strain	Phenotype	Source
E. coli HB101 harboring pBR322	Am^r, Tc^r, Rif^s	ATCC 35654 standard strain/ Bio Rad/ USA
E. coli MM 294	Rif^r, Am^s, Tc^s	ATCC 35545 Standard strain/ Bio Rad/ USA
Pseudomonas aeruginosa	Wild Type	Department of Biotehnology, Al-Nahrain University

Am: Ampicillin, Rif: Rifampicin, Tc: Tetracycline, r: resestance, s: sensitive

2.1.5 Media

2.1.5.1 Ready to use culture media:

These media were prepared according to the manufacturing companies and sterilized by autoclave at 121 ºC for 15 min.

Medium	Company/Origin
Brain heart infusion agar	Oxoid/England
Brain heart infusion broth	Oxoid/ England
MacConky agar	BDH/England
Muller Hinton Agar	Oxoid/ England
Nutrient agar	Difco/USA
Nutrient broth	Oxoid/ England

2.1.5.2 Laboratory prepared media

- **Luria-Bertani (LB) medium (Green and Sambrook, 2012)**

This medium was prepared by dissolving tryptone (10 g), yeast extract (5 g) and NaCl (5 g) in 950 ml D.W., pH was adjusted to 7, then the volume was completed to 1 L, sterilized by autoclaving.

2.1.6 Buffers and Solutions

2.1.6.1 Buffers and Solutions for extraction DNA:

Buffers and solution for extraction DNA were prepared according to Green and Sambrook (2012) as follows:

- **SET buffer**

This solution was prepared to consist of 75 mM NaCl, 25 mM EDTA and 20 mM Tris-HCl, pH was adjusted to 8 and sterilized by autoclaving.

- **Lysozyme solution (10mg/ml)**

It was freshly prepared by dissolving 10 mg of lysozyme in 1ml of sterilized distilled water.

- **Sodium dodecyl sulphate solution (10%)**

It was freshly prepared by dissolving 10 g of SDS in 100 ml of distilled water.

- **Sodium chloride solution (5M)**

It was prepared by dissolving 29.2 g of NaCl in 80 ml of distilled water, and then the volume was completed to 100 ml with distilled water and sterilized by autoclaving.

- **TE buffer**

It was prepared to consist of 10 mM Tris-HCl and 1mM EDTA, pH was adjusted to 8 and sterilized by autoclaving.

- **Solution I**

This solution was prepared to consists of 50 mM Glucose, 10 mM EDTA and 25 mM Tris-HCl, pH was adjusted to 8 and sterilized by autoclaving.

- **Solution I I**

SDS 1% solution was prepared in 0.2 N NaOH solution.

- **Potassium acetate solution**

Potassium acetate (5 M), pH was adjusted to 4.8 by glacial acetic acid and sterilized by autoclaving.

- **Sodium acetate (3 M**)

Sodium acetate (40.8 g) was dissolved in 80 ml of H_2O, pH adjusted to 4.8 with glacial acetic acid. The volume completed to 100 ml and dispensed into aliquots and sterilized by autoclaving.

- **STET**

It prepares in I litter and its component are:

8 % Sucrose

0.5 % Triton x- 100

50 m M EDTA (pH 8)

10 mM Tris-Cl (pH8)

2.1.6.2 Gel electrophoresis buffers and solutions

Gel electrophoresis buffers and solutions were prepared according to Green and Sambrook (2012) as follows:

- **Tris-Borate-EDTA (TBE) buffer (5 X)**

Tris-Borate-EDTA (TBE) Buffer (5 X) was prepared by dissolving 54 g Tris-HCl and 27.5 g boric acid in 900 ml distilled water, then 20 ml of 0.5 M EDTA was added. Volume was completed to 1000 ml with distilled water, pH was adjusted to 8 and sterilized by autoclaving.

- **Loading buffer (6X)**

This solution was prepared by dissolving 0.25 g of bromophenol blue and 40 g of sucrose in 80 ml of distilled water, then volume was completed to 100 ml with distilled water.

- **Ethidium bromide solution (10mg/ml)**

It was prepared by dissolving 1 g of ethidium bromide in 100 ml of distilled water and stirred on magnetic stirrer until completely dissolved

then filtered through a Whattman filter paper No.1 and stored in a dark bottle at 4°C until use.

2.1.6.3 Antibiotic solutions (Green and Sambrook, 2012)

- Ampicillin

It was prepared as stock solution (25 mg/ml) of Ampicillin Sodium salt in water, sterilized by filtration and stored at –20 °C until use.

- Tetracycline

It was prepared as stock solution (12.5 mg/ml) of tetracycline hydrochloride in ethanol/ water (50%, v/v), sterilized by filtration and stored at dark bottle at –20 °C.

- Rifampicin

It was prepared as stock solution (30 mg/ml) of Rifampicin Sodium salt in water, sterilized by filtration and stored at –20 °C until use.

- Cefotaxime

It was prepared as stock solution (10 mg/ml) of Cefotaxime Sodium salt in water, sterilized by filtration and stored at –20 °C until use.

2.1.6.4 Polymerase chain reaction (PCR) solutions

- **Genomic DNA Isolation (Qiagen Kit):** Qiagen Kit contains the following solutions:
  1. **Buffer ATL** (Bacterial lysis buffer).
  2. **Buffer AL** (Equilibration buffer).
  3. **Buffer AW1** (Wash buffer 1).
  4. **Buffer AW2** (Wash buffer 2).
  5. **Buffer AE** (Elution buffer).

6. **Proteinase K** (40 mg/ ml in distilled water).

- **PCR master mix (2 X) (Promega)**

DNA polymerase (5 U/µl)	2.5 U
dNTPs	2.5 mM each
PCR reaction buffer	1 X
Gel loading buffer	1 X

- **Primers (Sigma/USA)**

The following primers were used in this study:

Primer name	**Primer sequence 5'----3'**	**Product size**	**References**
E. coli lacZ Forward	ATGAAAGCTGGCTACAGGAAGGCC	264bp	(Corinne, 2004)
E. coli lacZ Reverse	GGTTTATGCAGCAACGAGACGTCA		
P. aeruginosa 16S rRNA gene Forwared	GGGGGATCTTCGGACCTCA	420bp	(Spilker *et al.*, 2004)
P. aeruginosa 16S rRNA gene Reverse	TCCTTAGAGTGCCCACCCG		

2.1.6.5 Transformation solutions (Green and Sambrook, 2012)

- $MgCl_2$-$CaCl_2$ solution (80 mM, 20 mM) in distilled water.
- $CaCl_2$ (0.1 M) in distilled water.

2.1.6.6 Conjugation Solution (Weiservar *et al.* 1987)

- $MgCl_2$ solution (10 mM) in distilled water .

2.2 Methods:

2.2.1 Sterilization methods (Green and Sambrook, 2012)

- **Moist heat sterilization (Autoclaving)**

Media, buffers and solutions were sterilized by autoclaving at 121°C and 15 Ib/in^2 for 15 minutes, except some heat sensitive solutions.

- **Dry heat sterilization**

Electric oven was used to sterilize glassware and some other tools at 180°C for 2 hours.

- **Membrane sterilization (Filtration)**

Millipore filter unit was used to sterilize heat sensitive solutions by using millipore filters (0.45 µm).

2.2.2 Maintenance of bacterial isolates

Maintenance of bacterial isolates was performed according to Green and Sambrook (2012)

- **Short-Term storage**

Bacterial isolates were maintained for few weeks on nutrient agar slant. They were tightly wrapped with parafilm, and then stored at 4°C.

- **Medium- Term Storage**

Bacterial isolates were maintained as stab cultures for few months. Such cultures were prepared in small screw-capped bottles containing 2-3 ml of nutrient agar medium and stored at 4°C.

- **Long-Term Storage**

Single colonies were cultured in nutrient broth and incubated for 24 h at 37 Cº, and then 8.5 ml of bacterial culture mixed with 1.5 ml of glycerol, and stored for a long time

2.2.3 Characterization of nanoparticles:

- **UV–visible Spectroscopy (Rajesh *et al.*, 2010):**

In this technique the nanoparticles in suspension was determined, by measuring the peak shifts of titanium dioxide and zinc oxide nanoparticles, in which appropriate dilutions were prepared from stock solution and read in UV –spectrophotometer (UV-visibl Spectrophotometer) at wavelength between 300 to 750 nm.

- **Transmission Electron Microscopy (SEM) (Elumalai *et al*., 2010):**

Transmission electron microscopy offer nanometer resolution for measuring nanoparticle (TiO_2 and ZnO) size. Aliquots of 50 µl of nanoparticles samples were deposited on glass cover slips. After being air dried for 1 hr. The cover slips were mounted on TEM tubs, Digital images of nanoparticle samples were acquired using a transmission electron microscope.

2.2.4.1 Antibiotic susceptibility test (Atlas *et al.*, 1995)

Disc diffusion method was used to investigate antibiotic sensitivity of the bacterial isolates. A sterile cotton swab was dipped into inoculums of fresh cultures of each bacterial isolates, and the entire surface of the Mullar Hinton agar plates was swabbed three times by rotating the plate approximately 60º between streaking to ensure even distribution of inoculums. The inoculated plates were kept at room temperature for 10

min. to allow the absorption of excess moisture, and then discs of antibiotics were applied and incubated at 37°C for 24 hrs. The diameters of the inhibition zones (clear area around discs) were measured in mm. and compared with that of standards of the National committee for clinical laboratory standards (NCCLs, 2011).

2.2.4.2 Minimum inhibitory concentration of nanoparticles (Saxena *et al.*, 1995).

In order to determine the MIC of nanoparticals, a series of test tubes containing 5 ml of nutrient broth supplemented with different concentrations of nanoparticals (10, 5, 3.5, 0.35, 0035, 0.0035, 0.00035 mg/ml for TiO_2 and 10, 5, 1.5, 0.5, 0.05, 0.005, 0.0005 mg/ml for ZnO NPs). The tubes were inoculated with portions of 100 µl of fresh culture of each isolate and incubated at 37ºC for 24 hrs, with shaking. Aliquots (100 µL) of appropriate dilution of each culture were spread on brain-heart infusion agar plates and incubated at 37ºC for 24 hrs. The lowest concentration of the nanoparticles (ZnO or TiO_2) that inhibits the growth of bacterial isolates considered as the minimum inhibitory concentration (MIC).

2.2.4.3 Antibacterial activity of nanoparticles:

The cup-plate agar diffusion method was used as described by Xie *et al*. (2011) to test the antibacterial activity of the nanparticles. Fifty milliliter of sterile nutrient agar were inoculated with 0.5 ml of bacterial suspension, mixed gently, then distributed into sterile petri dishes. The agar was left to set and in each of these plates, 1 cup, 10 mm in diameter, was cut using a sterile cork borer No. 4 and the agar discs were removed. Alternate cups were filled with 50 µl of different concentration of ZnO or TiO_2 nanoparticles using micropipette and allowed to diffuse at room

temperature for an hour. The plates were then incubated in the upright position at 37ºC for 18 hours. Two replicates were carried out for each nanoparticals against each of the test organism. After incubation the diameters of the growth inhibition zones were measured.

2.2.5 Extraction of DNA

2.2.5.1 Salting out method:

Genomic DNA was extracted according to salting out method described by Kieser (1995), as follows:-

1- Twenty ml of fresh culture for the selected isolate, grown in brain heart infusion broth at 37 ˚C for 24 hrs, was pelleted by centrifugation at 6000 rpm for 15 min.
2- The pellet was washed with 3 ml of SET buffer, centrifuged and then cells were resuspended in 1.6 ml of SET buffer. Freshly prepared lysozyme (final concentration 1 mg /ml) was added, and incubated at 37˚C for 30 min.
3- One ml of 10% SDS was added, mixed by inversion, and then incubated at room temperature for 15 min.
4- Two ml of 5 M NaCl was added and mixed by inversion at room temperature.
5- An equal volume of chloroform was added, mixed by inversion for 15 min. Then centrifuged (6000 rpm. at 4˚C) for 15 min
6- The aqueous phase (upper) was transferred to another sterile eppendorff tube, and 0.6 volume of isopropanol was added, mixed by inversion, and kept at room temperature for 5 minutes.
7- Eppendorff tubes were centrifuged at 13000 rpm for 15 min at 4˚C.
8- The isopropanol layer was discarded gently and the precipitated DNA was dissolved in 100 µl of TE buffer and stored at -20 ˚C.

- **Salting out with nanoparticles:**

Genomic DNA was extracted using modified salting out method as follow:-

1- Twenty ml of fresh culture for the selected isolate, grown in brain heart infusion broth at 37 ºC for 24hrs, was pelleted by centrifugation at 6000 rpm for 15 min.
2- The pellet was washed with 2 ml of nanoparticle solution [ZnO (0.4 mg/ml), or TiO_2 (0.2 mg/ml)], centrifuged and then the cells were resuspended in 1.5 ml of nanoparticles solution, mix by inversion and incubated at 37ºC for 10 min.
3- The mixture then sonicated for 4 min at 4 ºC using 60 KH_z. the sonication was done for 2 cycles' 2 min sonication then 2 min brake.
4- The subsequent steps were done from step four as in item 2.2.5.1.

2.2.5.2 Alkali lysis method:

Plasmid DNA was extracted by alkali lysis method described by Green and Sambrook (2012) as follows:-

1- Fifty milliliter of fresh culture was centrifuged, then resuspended in 1 ml of Solution I containing freshly prepared lysozyme (final concentration 10 mg/ ml), and incubated at 37 ºC for 30 min.
2- One ml of 10% SDS was added, mixed by inversion, let stand at room temperature for 5 min.
3- Two milliliter of freshly prepared solution II were added, then the top of the tube was covered with the para-film and the contents were mixed by gently inverting the tube several times, then let stand on ice for 10 min.
4- One and a half ml of an ice-cold solution of a 5 M Potassium acetate (pH 4.8) was added, the top of the tube was covered with the para-film and

the contents were mixed by inverting the tube sharply several times, then let stand on ice for 10 min.

5- The tube was centrifuged at 6000 rpm for 20 min at 4 Cº. The bacterial debris should form a tight pellet on the bottom of the tube.
6- The supernatant was transferred to another tube and add 0.6 volume of isopropanol to the tube, mixed well and let stand at room temperature for 15 min.
7- The DNA was recovered by centrifugation at 12000 rpm for 30 min. at room temperature (salt may precipitate if centrifugation is carried out at 4 Cº).
8- The supernatant was discarded and the pellet was washed with 70 % ethanol at room temperature, discarded as much ethanol as possible, then let to dry.
9- The pellet was dissolved in a total volume of 800 µl of TE (pH 8)**.**

- **Alkali lysis with nanoparticles:**

The alkali lysis method was modified by adding 1ml of nanoparticles ZnO (0.4 mg /ml) or TiO_2 (0.2 mg /ml) in addition to 1 ml of solution I in step 1.

2.2.5.3 Boiling method:

Plasmid DNA was extracted using boiling method described by Green and sambrook (2012):

1. Five milliliter of an overnight culture was harvested in 1.5 ml microcentrifuge tube by centrifugation at 13000 rpm for 1 min.
2. Supernatant was discarded and pellet was resuspended in 350 µl of STET solution. Twenty five µl of freshly prepared solution of lysozyme (10 mg/ml in 10 mM Tric-Cl pH 8.0) were added, and incubated at 37 ºC for 15 min.

3. The contents were mixed by vortexing for 3 sec. The tube was placed in boiling water bath for exactly 40 sec.
4. Centrifuged immediately for 10 min. at room temperature in a microcentrifuge. Pellet was removed from the microcentrifuge tube using a tooth pick, then 40 µl of 3 M sodium acetate and 420 µl of isopropanol were added.
5. The content were mixed by vortexing and stored for 15 min in ice bath. The tube centrifuged for 15 min at 13000 rpm.
6. DNA pellet was dried and resuspended in 30 µl of TE buffer pH 8.0.

- **Boiling method with nanoparticles**:

Boiling method was modified to extract plasmid DNA in the presence of nanoparticals. The modification was made in step 2. In addition to STET solution and lysozyme, 100 µl of nanoparticles ZnO (final concentration 0.4 mg /ml) or TiO_2 (0.2 mg /ml) were added and incubated at 37 ºC for 15 min.

2.2.6 Polymerase chain reaction:

2.2.6.1 DNA Extraction Kit (Qiagen kit)

This protocol is designed for isolation of genomic DNA from Gram-negative bacteria to use it in PCR amplification:

1- Five milliliter of an overnight culture was harvested in a microcenterifuge tube by centrifuging for 10 min at 7500 rpm.
2- Supernatant was discarded and pellet was resuspended in 180 µl of buffer ATL (2.1.6.4).
3- Twenty microliter of proteinase K (2.1.6.4) was added, then mixed thoroughly by vortexing, and incubated at 56 °C for 1hr. Vortex occasionally during incubation to disperse the sample.

4- Vortexing for 15s. Were made, then 200 µl of buffer AL (2.1.6.4) was added to the sample, mixed thoroughly by vortexing. Then 200 µl of ethanol (96-100%) were added, and mixed again thoroughly by vortexing.

5- The mixture from step 4 was pipetted (including any precipitate) into the DNeasy mini spin column and placed in 2 ml collection tube. Then it was centrifuged at 8000 rpm for 1min.The flow-through and collection tube were discarded.

6- The DNeasy mini spin column placed in a new 2 ml collection tube, then 500 µl of buffer AW1(2.1.6.4) was added, and centrifuge at 8000 rpm for 1min discard flow-through and collection tube.

7- The DNeasy mini spin column was placed in a new 2 ml collection tube, and 500 µl of buffer AW2 (2.1.6.4) was added, centrifuge for 2 min. at 14,000 rpm to dry the DNeasy membrane. The flow-through and collection tube were discarded.

8- The DNeasy mini spin column was placed in a clean 1.5 ml or 2 ml microcentrifuge tube, and pipetted 200 µl of buffer AE (2.1.6.4) directly to the DNeasy membrane. Then incubated at room temperature for 1min, and centrifuge for 1min at 8000 rpm to elute.

9- The previous step (8) was repeated for maximum DNA yield.

2.2.6.2 Preparation of primers solution:

The lyophilized primer was dissolved using deionizer distil water DDH_2O to obtain 100 pmol/µl in the master tube, then 10 pmol/µl was prepared as a working solution by taking 10 µl from the master tube and the volume was completed to 100 µl by adding DDH_2O.

2.2.6.3 Amplification of DNA

2.2.6.3.1 Amplification of lacZ gene of *E. coli* HB 101:

The lacZ gene of *E. coli* was amplified by using specific primer for this gene (2.1.6.4) (Corinne, 2004).

The PCR reaction was performed by adding the following:

PCR reaction	50 µl Rxn
2x PCR master mix solution	25 µl
Template DNA (genomic DNA)	1-2 µl
Primer (F:10 pmol/µl)	1 µl
Primer (R: 10 pmol/µl)	1 µl
Distilled water	21-22 µl
Total reaction volume	50µl

Amplification was carried out according to the following thermal and cycling condition:

	Temp.	Time	Cycle No.
Initial denaturation	94◦C	3 min	1
Denaturation	94◦C	1 min	35
Annealing	55◦C	1 min	
Extension	72◦C	1min	
Final extension	72◦C	10min	1

- In order to study the effect of nanoparticles on PCR reaction many experiments were done, by manipulating with time at first and with

the number of cycle secondly , in which 1-2 µl of nanoparticles (ZnO (0.4 mg/ml) or TiO_2 (0.2 mg/ml) were added to PCR reaction and D.W. was reduced to about 20-21 µl.

- PCR program and conditions were modified when using nanoparticles mixture, The final modified program is as follows:

	Temp.	Time	Cycle No.
Initial denaturation	94◦C	1.5 min	1
Denaturation	94◦C	30 sec.	28
Annealing	55◦C	1 min.	
Extension	72◦C	30 sec.	
Final extention	72◦C	7 min.	1

2.2.6.3.2 Amplification of 16S RNA of *P. aeruginosa*.

The 16S r RNA gene of *Pseudomonas aeruginosa* was amplified using specific primer (Spilker *et al.*, 2004). The reaction mixture was of the same composition as described above. The conditions of this set of primer were as following:

	Temp	Time	Cycle No.
Initial denaturation	95◦C	3 min	1
Denaturation	95◦C	1 min	40
Annealing	58◦C	1 min	
Extension	72◦C	2min	

Final extension	72◦C	8min	1

- The effect of nanoparticles on PCR reaction was studied as mentioned above (with lacZ gene of *E. coli*)
- PCR program and conditions were modified when using nanoparticles. The final modified program is as follows:

	Temp	Time	Cycle No.
Initial denaturation	95◦C	1.5 min	1
Denaturation	95◦C	30 sec.	30
Annealing	58◦C	1 min	
Extension	72◦C	30 sec.	
Final extension	72◦C	5min	1

The PCR products were examined by agarose gel electrophoresis.

2.2.7 Measuring the purity and concentration of DNA (Green and Sambrook, 2012):

Purity and concentration of DNA solution was measured by using Nanodrop spectrophotometer, by using 1µl of each DNA sample according to the nanodrop spectrophotometer manual, and the absorbency at 260 and 280 nm was measured after calibration with D.W. or TE buffer at 260 nm and 280 nm respectively. For PCR products, the two dyes of the GoTaq green master mix interfere with readings. Thus, the colorless master mix was used instead. Pure DNA has an A_{260}/A_{280} ratio of 1.7-1.9. The concentration of double strand DNA (ng/µl) was measured directly by the device.

2.2.8 Agarose gel electrophoresis (Green and Sambrook, 2012)

Agarose gel (0.7-1.5 %) was utilized to detect the size of genomic DNA bands and PCR products. Gel was run horizontally in 1 X TBE buffer. Electrophoretic buffer was added to cover the gel. Samples of DNA were mixed with loading buffer (1:10 v/v) and loaded into the wells and run for 1-3 hours at 5 V/cm, then agarose gel was stained with ethidium bromide by immersing in distilled water containing the dye at a final concentration of 0.5 µg/ml for 30-45 minutes. DNA bands were visualized by UV transilluminator. Gel was de-stained using distilled water for 30- 60 min. to get rid of background before photographing of DNA bands.

2.2.9 Transformation (Green and Sambrook, 2012)

Attempts were done to transform *E. coli* MM294 (Rif^r, Am^s, Tc^s) with DNA of *E. coli* HB101 (Am^r, Tc^r, rif^s), and another attempts were done to transform *E. coli* MM294 that rifampicin resistance and tetracycline sensitive with DNA of *P. aeruginosa* that resistance to tetracycline and sensitive to rifampicin, as follow:

1. One hundred milliliter of nutrient broth were inoculated with a single colony of *E. coli* MM294, the culture was incubated for 3 hrs at 37 C^o with vigorous agitation.
2. The bacterial cells were transferred to sterile, disposable ice cold (50 ml) falcon tube, and the culture was cooled to 0 C^o by storing the tubes on ice for 10 minutes.
3. The cells were recovered by centrifugation at 6000 rpm for 15 min at 4 C^o.
4. The pellet was resuspended by gentle vortexing in 30 ml of ice cold [$MgCl_2$-$CaCl_2$ (80 mM, 20 mM)].

5. The cells were recovered by centrifugation at 6000 rpm for 10 min at 4 Cº.

6. The pellet was resuspended by gentle vortexing in 2 ml of ice cold 0.1 M $CaCl_2$ for each 50 ml of original culture.

7. Two hundred microliters of each suspension of competent cells were transferred to sterile tube using a chilled micropipette tip, DNA (10 µl) was added to each tube; the tubes were mixed by swirling gently, and stored on ice for 30 minutes.

8. The tubes were transferred to rack, placed in preheated 42 Cº circulating water bath. Then, the tubes were stored in rack for 90 seconds (the tubes should not be shaken).

9. The tubes were transferred rapidly to an ice bath; the cells were allowed to chill for 1-2 minutes.

10. Nutrient broth medium (800 µl) was added to each tube, the cultures incubated for 45 min. at 37 Cº to allow bacteria to recover and to express the marker encoded by DNA.

11. Then 0.1 ml samples of the transformation mixture were diluted properly and plated on to nutrient agar medium containing 15 µg/ ml ampicilin and 10 µg/ml tetracycline. The plates were inverted and incubated at 37 Cº for 18 hrs.

12. Two controls were made. In the first, 10 µl of DNA was added to 100 µl of TE buffer, while in the second 10 µl of TE buffer was added to 100 µl of cell suspension of competent cells and spreaded on selective medium (nutrient agar containing 15 µg/ ml ampicilin and 10 µg/ ml tetracycline) to select transformants of *E. coli* MM294 with DNA of *E. coli* HB 101. Another selective medium is nutrient agar containing 10 µg/ ml tetracycline to select transformants of *E. coli* MM294 with DNA of *P. aeruginosa*. The plated was incubated at 37 Cº for 18 hrs.

- **Transformation with nanoparticles:**

To study the effect of nanoparticles on the transformation experiment, the same procedure (described above) was used except for two attempts of the modification which were performed in step 4. In the first attempt 10 ml of nanoparticles [ZnO (0.4 mg/ml), TiO_2 (0.2 mg/ml)] were added to 30 ml of cells suspension in ice cold $MgCl_2$ - $CaCl_2$. In the second attempt, the pellet was resuspended in 10 ml of nanoparticle solutions only.

2.2.10 Conjugation:

Conjugation was done between *E. coli* HB101 (Am^r, Ctx^s) and *P. aeruginosa* (Am^s, Ctx^r). Many experiments were performed according to Weiservar *et al.* (1987) and modified by using nanoparticles as follow:

1- The two bacterial strains were grown in 5 ml of LB broth and incubated at 37 Cº for 4 hrs.

2- The two cultures were mixed and incubated at 37 Cº for 3 hrs, then centrifuged at 6000 rpm for 15 min.

Harvested cells were washed and resuspended in 5ml of 10 mM $MgSO_4$. **The modification was to wash and resuspend cells in 5 ml of nanoparticles [ZnO (0.4 mg/ml) or TiO_2 (0.2 mg/ml)] instead of $MgSO_4$ solution.**

3- The cell suspention was filtered using Milipore filter (0.45µm), then the filter paper was plated on LB agar and incubated at 37 Cº for 18 hrs.

4- The filter paper was removed and washed the growth with 2 ml of LB broth, then vortexed in order to get a homogenized suspension.

5- Appropriate dilution were plated on selective LB plates (LB supplemented with 10 µg/ml cefotaxime and 10 µg/ml tetracycline), that

allowed the growth of transconjugant colonies, but not recipient neither of the donor colonies.

6- Each of bacterial strain (controls) was plated onto the same selective medium to account for spontaneous mutants or experimental errors.

7- Conjugation frequency was expressed as the number of transconjugant per recipient cells, in mating mixture.

Chapter three

Results
And
Discussion

3. Results and Discussion

3.1 Characterization of TiO_2 and ZnO colloid nanoparticles:

Zinc oxide and TiO_2 colloid nanoparticles are the most popular and widely used in medicine, in industry and in cosmetic and they have low toxicity effect. So they have been chosen in this study.

They are characterized through the measurement of their wavelength using UV\vis spectrophotometer, hence the nanoparticles have high sensitivity to light which led to change in the orbital motion leading to a change in the physical and chemical properties of TiO_2 and ZnO nanoparticles (Ahmed *et al*., 2011).

Results in figure (3-1) and (3-2) showed that the highest pecks were 450 nm for TiO_2 and 400 nm for ZnO, and this indicates that these nanoparticles had high purity with no changing in its physical or chemical properties.

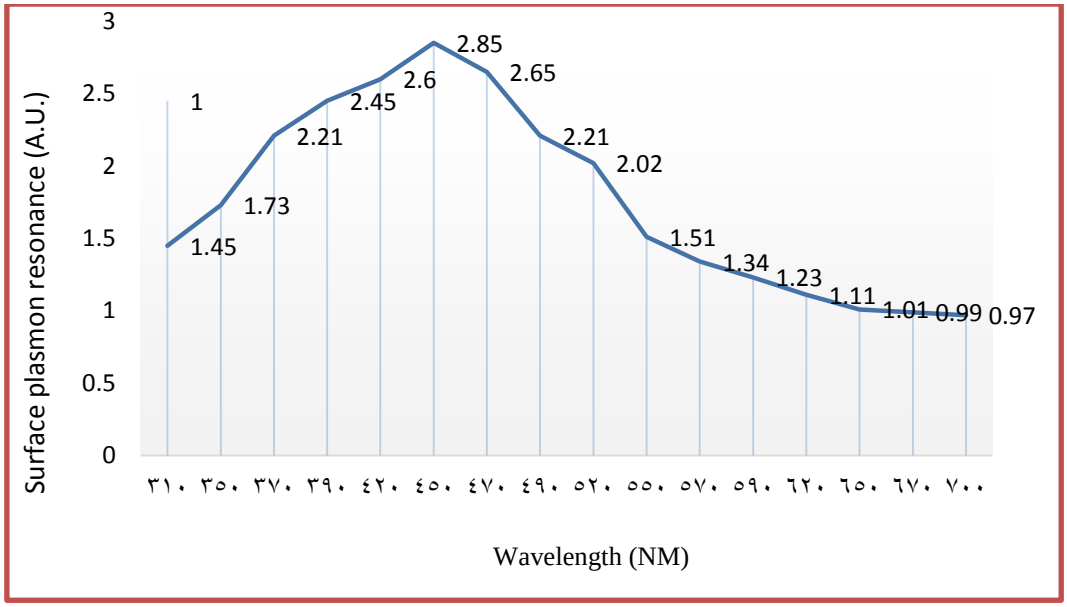
Other properties (color, shape and size) of particles were studied to characterize the nanoparticles. As mentioned by the manufactured company, TiO_2 nanoparticle was a white solution and a mixture of rutile and anatase with size (50-150) nm, and ZnO nanoparticle was a pale yellow solution with size (20-50) nm and anatas phase.

The minerals rutile and brookite as well as anatase all have the same chemistry, but they have different structures. At higher temperatures, anatase will automatically revert to the rutile structure. Rutile is the more common and the more well known mineral of the three, while anatase is the rarest. Anatase shares many of the same or nearly the same properties as rutile such as luster, hardness and density. However due to structural differences anatase and rutile differ slightly in crystal habit and more

distinctly in cleavage. Crystals of anatase are very distinctive and are not easily confused with any other mineral (Zhang *et al*., 2011).

Analysis of the nanoparticles by transmission electron microscope illustrated that titanium dioxide nanoparticles are predominantly spherical and the average diameter was estimated to be about 100 nm (figure 3-3B), while the zinc oxide was about 35 nm in size. Their shape is also spherical (figure 3-3 A). These results confirmed the nanoparticle properties as mentioned by the company.

It was demonstrated that some properties of TiO_2 and ZnO are very affected with its structure either anatase or rutile . Since the anatase phase is chemically and optically active, it is suitable for catalysts and supports. Usually, the anatase phase can be transformed into rutile via subjecting to light. This anatase-to-rutile phase transition, and subjecting to light induces the variation of the optical properties of TiO_2 and ZnO nanoparicals. So the temperature and light should be a controlled in order to make desirable properties of TiO_2 and ZnO for various applications (Asthanal *et al.*, 2010).



Figur (3-1): Characterization of TiO_2 colloid nanoparticles by spectrophotometer.

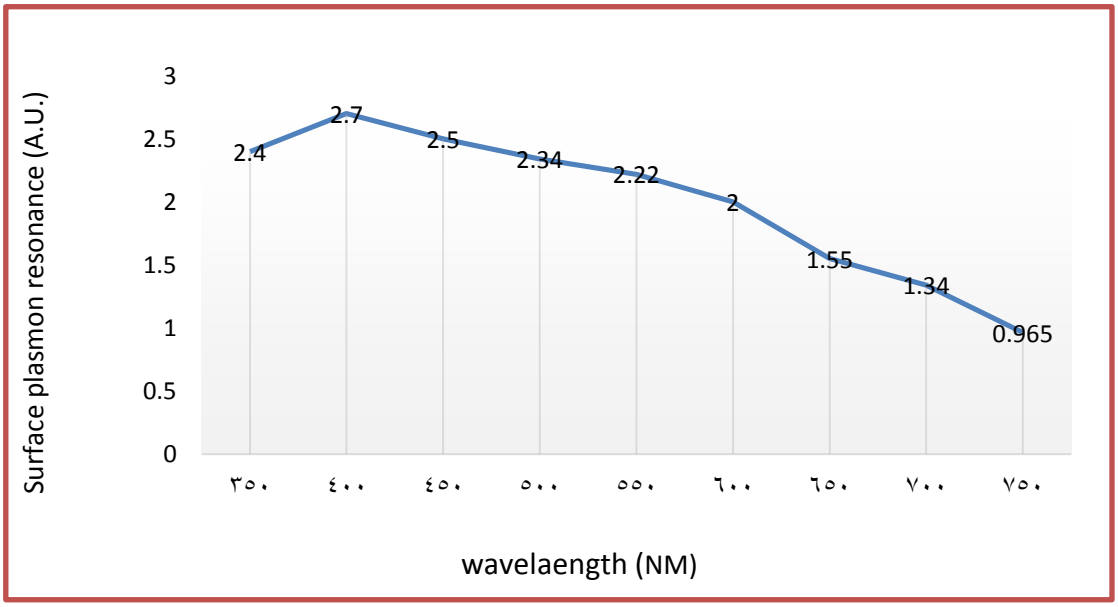


Figure (3-2): Characterization of ZnO colloid nanoparticles by spectrophotometer.

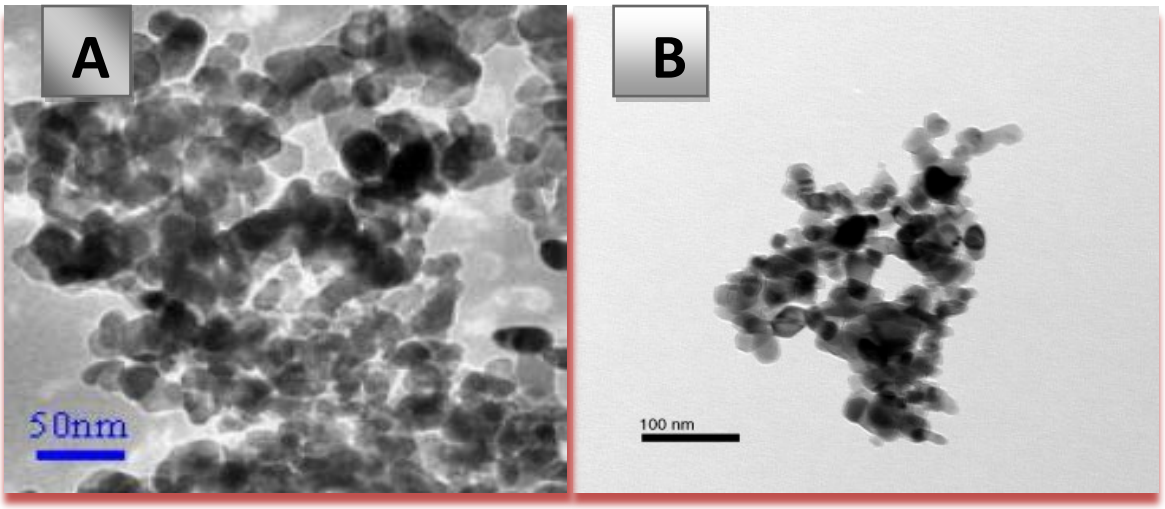


Figure (3-3): Transmission electron microscope analysis for ZnO and TiO_2 nanoparticles (A) Zinc oxide Nanoparticle, (B) Titanum dioxide nanoparticle. (Sigma, USA)

3.2.1 Antibiotic Susceptibility:

The standard disk diffusion method was used to determine the susceptibility of *E. coli* and *P. aeruginosa* to different antibiotics, in

order to confirm their sensitivity and resistant to antibiotics to chose markers for transformation and conjugation experiments. Results in table (3-1) showed that *E. coli HB101* was resistant to ampicillin and tetracycline, while *E. coli MM294* was resistance to rifampicine. Which confirmed their resistance to these antibiotics. Results demonstrated in table (3-1) showed also that *P. aeruginosa* was resistant to ampicillin, amoxicillin, cephradin, penicillin, cephalothin, cephotaxime, tetracycline, vancomycine, clindamycin, azithromycin and naldixic acid, while it was sensitive to chloramphenicol, doxycycline, ciprofloxacin, norfloxacillin, novobiocine and rifampicin. In general it was documented that very few of the conventional antibiotics are active against this isolate of *P. aeruginosa* (four antibiotics only). Many studies demonstrated that *P. aeruginosa* isolate are resistance to multiple antibiotics, these isolates have multiple mechanisms for antibiotic resistance like inactivation of antibiotics by enzymes, modification of target site, impaired of penetration of drug target (Khan *et al*., 2000). Table (3-1) showed that *E. coli* HB101 was resistant to ten of the tested antibiotics, and sensitive to eight. On the other hand *E. coli* MM294 was resistant to two antibiotics and sensitive to the other sixteen antibiotics, while *P. aeruginosa* was resistance to twelve antibiotics and sensitive to four. Resistance to antibiotics may be attributed to the antibiotic resistance genes; some of these genes may be located on chromosomal DNA, while others may be located on plasmid DNA (Jawetz *et al*., 1998). The resistance of bacteria to several antibiotics could be due to the permeability of the outer membrane, which might prevent the entery into the cell of most of these antibiotics or due to certain mutations that occur as a result of overuse and misuse of antibiotics (Stock and Wiedemann, 2001)

Table (3-1) Antibiotic susceptibility of *E. coli* and *P. aeruginosa* isolates.

Antibiotics	Code	*E. coli* HB101	*E. coli MM294*	*P. aeruginosa*
Amikacin	AK	R	S	R
Amoxicillin	AX	R	S	R
Ampicillin	AM	R	S	R
Azithromycin	AZM	S	S	R
Cefotaxime	CTX	S	S	R
Cephalothin	KF	R	S	R
Cephradine	CE	S	S	R
Chloramphenicol	C	S	S	S
Ciprofloxacin	CIP	R	S	S
Clindamycin	DA	R	S	R
Doxycycline	DO	S	S	S
Nalidixic acid	NA	R	S	R
Norfloxacillin	NOR	S	S	S
Penicillin	P	R	S	R
Rifampicin	RA	S	R	S
Tetracycline	TE	R	S	R
Vancomycin	VA	R	S	R
Novbiocine	NOV	S	R	S

S: sensitive, R: resistant

3.2.2 Antibacterial activity of nanoparticles:

The effect of nanoparticle (TiO_2 and ZnO) on bacterial growth was tested. Results showed that nanoparticles had different antibacterial

effect on gram negative bacteria (*E. coli* HB 101, *P. aeruginos*a). TiO_2 nanoparticles with concentration 4 mg\ml had slight effect on *E. coli* HB 101 and *P. aeruginosa* with inhibition zone 6 mm, while 3 mg/ml of ZnO nanoparticles had significant effect on both bacteria with inhibition zone 20-25 mm (figure 3-4).

Zinc oxide and TiO_2 nanoparticles showed antimicrobial effect on the tested bacteria, this effect may occur due to the interaction with the cell wall of bacteria which lead to the formation of pores in these walls. Sondi *et al.* (2004) and Zhang *et al*. (2011) referred that accumulation of the ZnO nanoparticles in the pits caused the permeability of the cell membrane. Other reason caused the killing of the bacterial cells may be attributed to the effect of nanoparticles on the proteins in the cytoplasm of the cells, which lead to regulation of functions in the cells. The antimicrobial activity of the nanoparticles is known to be a function of the surface area in contact with the microorganisms. Large surface area of the nanoparticles (the small size and the high surface to volume ratio) enhances their interaction with the microbes to carry out a broad range of probable antimicrobial activities (Gutierrez *et al*., 2010).

The inhibitory activity of TiO_2 was due to the photocatalytic generation of strong oxidizing power when illuminated with UV light. The generation of active free hydroxyl radicals (- OH) by photo excited TiO_2 particles is probably responsible for the antimicrobial activity. Nanostructured TiO_2 on UV irradiation can be used as an effective way to reduce the disinfection time, eliminating pathogenic microorganisms in food contact surfaces and enhance food safety. In recent years, visible light absorbing photocatalysts with Ag, ZnO and

TiO_2 has proved to be successful at killing *Pseudomonas,* and *E. coli* (Chorianopoulos *et al.,* 2011).

There are several mechanisms which have been proposed to explain the antibacterial activity of nanoparticles. The generation of hydrogen peroxide from the surface of nanoparticles is considered as an effective mean for the inhibition of bacterial growth (Yamamoto, 2001). Another possible mechanism for ZnO antibacterial activity is the release of Zn^{+2} ions which can damage cell membrane and interact with intracellular contents (Brayner *et al*., 2006).

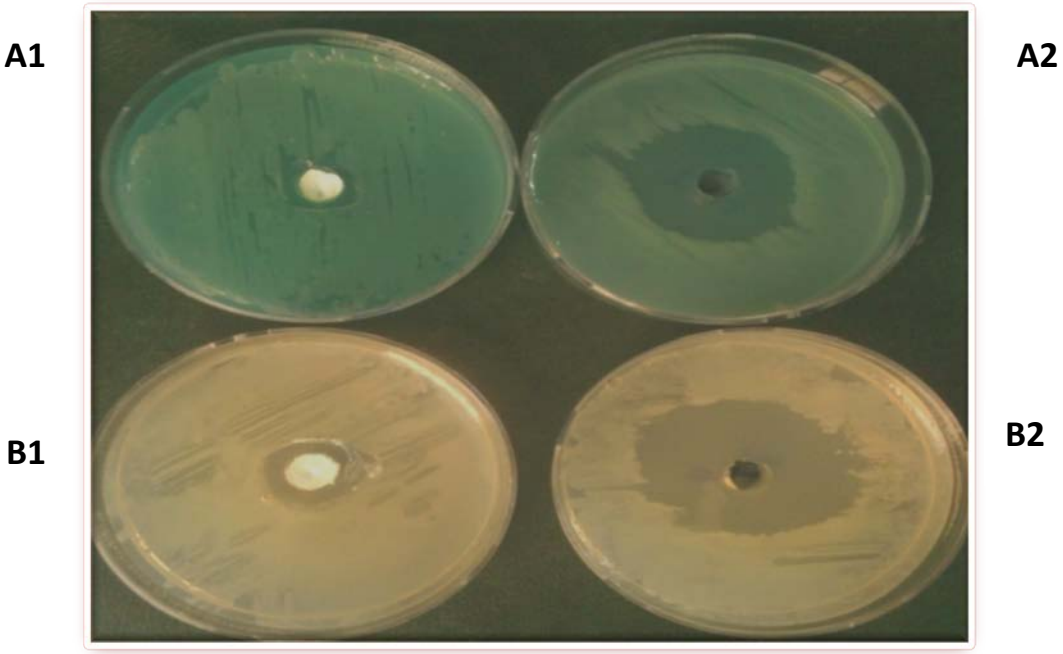


Figure (3-4): The effect of colloid NPs on bacterial growth after incubation at 37C° for 24 hrs. (A1) effect of TiO_2 on *P. aeruginosa*. (A2) effect of ZnO on *P. aeruginosa*. (B1) effect of TiO_2 on *E. coli* HB101 (B2) effect of ZnO on *E. coli* HB101.

3.2.3 Minimum inhibitory concentration of nanoparticles:

The effect of different concentrations of nanoparticles on *E. coli* HB101, *E. coli* MM294 and *Pseudomonas aeruginosa* was tested.

Results (table 3-2 a, b) show that high concentration of both nanoparticles (ZnO and TiO_2) which inhibited the growth of all tested bacteria. ZnO had high significant effect on bacterial growth. It was inhibited the growth of *E. coli* HB 101 and *E. coli* MM294 with the concentration of 10, 5, 1.5, 0.5 and 0.05 mg/ml, while the growth of *P. aeruginosa* inhibited with the concentrations of 10, 5, 1.5, and 0.5 mg/ml (table 3-2a).

Table (3-2a): The effect of different concentrations of ZnO nanoparticles on bacterial growth.

Concentration of ZnO NPs (mg\ml)	**Bacterial growth**		
	***E. coli* HB 101**	***E. coli* MM 294**	***P. aeruginosa***
10	-	-	-
5	-	-	-
1.5	-	-	-
0.5	-	-	-
0.05	-	-	+
0.005	+	+	++
0.0005	++	++	+++

(-) No growth, (+) Slightly growth, (++) Moderate growth, (+++) Good growth.

Result declared that TiO_2 had a high significant effect on bacterial growth. It was inhibited the growth of *E. coli* HB 101, *E. coli* MM294 and *P. aeruginosa* with the concentrations of 10, 5, 3.5, 0.35 mg/ml (table 3-2b).

Several mechanisms were proposed to explain the effect of nanoparticles on bacterial growth as mentioned in item (3.2.2).

Table (3-2b): The effect of different concentrations of TiO_2 nanoparticles on bacterial growth.

Concentration of TiO_2 NPs (mg\ml)	Bacterial Growth		
	***E. coli* HB101**	***E. coli* MM 294**	***P. aeruginosa***
10	-	-	-
5	-	-	-
3.5	-	-	-
0.35	-	-	-
0.035	+	+	+
0.0035	++	++	++
0.00035	+++	+++	+++

(-) No growth, (+) Slightly growth, (++) Moderate growth, (+++) Good growth.

Studies demonstrated that the nanosize of the nanoparticles assures large surface contact area with the microbial cells and, hence, a better interaction with the microbial target will occur, beside that the antibacterial efficacy of the nanoparticles depends on the shape of nanoparticles (Pal *et al.,* 2007). Other study demonstrates that the antibacterial activity of nanoparticles is dependent on the concentration of nanoparticles (Costescu *et al*., 2013).

3.3 Effect of nanoparticles on the DNA extraction:

One of the major problems associated with studying genes and their expression is the difficulty to obtain adequate and pure nucleic acid

samples. Some of the most difficult contaminants DNA are humic acids and proteins. Other difficulties are enzyme, consuming time for extraction and using different organic materials. Using nanoparticles proved to be powerful and decreasing the time, the number of chemicals needed for DNA purification. Moreover, it's not toxic and cheep.

In this study, the quality and quantity of isolated genomic DNA using nanoparticles (ZnO and TiO_2) was compared with that without using these nanoparticles. Genomic DNA was extracted from *E. coli* HB 101 and *P. aeruginosa* using a number of popular manual techniques (boiling method, alkali lysis and salting out methods).

Many attempts were made to examine different concentration of nanoparticles ranging from 0.1 to 1 mg/ml in different steps of these methods. It was found that the best results were obtained when nanoparticles used in first steps and in concentration of 0.2 mg/ml for TiO_2 and 0.4 mg/ml for ZnO for all DNA extraction methods (as mentioned in 2.2.5).

- Boiling method:

Results (table 3-3 and figure 3-5) pointed improvement of the quality of DNA, while the purity of extracted DNA from *E. coli* HB 101 and *P. aeruginosa* was 1.6 and became 1.7 to 1.8 when DNA extracted with nanoparticles.

The results have also showed that the concentration of DNA highly increased when DNA was isolated with ZnO nanoparticles from both bacteria. The concentration increased from 104.8 to 355 ng/µl for *E. coli* DNA and from 88 to 278 ng/µl for *P. aeruginosa* DNA, while the

concentration of DNA was not change when isolated with TiO_2 nanoparticles for both bacteria (table 3-3).

Table (3-3): Concentration and purity of DNA extracted from *E. coli* and *P. aeruginosa* by using boiling method.

Extracted DNA From	DNA concentration ng\µl	Purity of DNA (260 /280)
E. coli HB101	104.8	1.6
E. coli HB 101 with ZnO (0.4 mg/ml)	355	1.8
E. coli HB 101 with TiO_2 (0.2 mg/ml)	106	1.7
P. aeruginosa DNA.	88	1.6
P. aeruginosa with ZnO (0.4 mg/ml)	278	1.7
P. aeruginosa with TiO_2 (0.2 mg/ml)	90	1.7

Results illustrated in table (3-3) which shows also that extracted DNA by boiling method with using ZnO nanoparticles in concentration of 0.4 mg/ml increased the yield of isolated DNA about 3 to 3.4 folds.

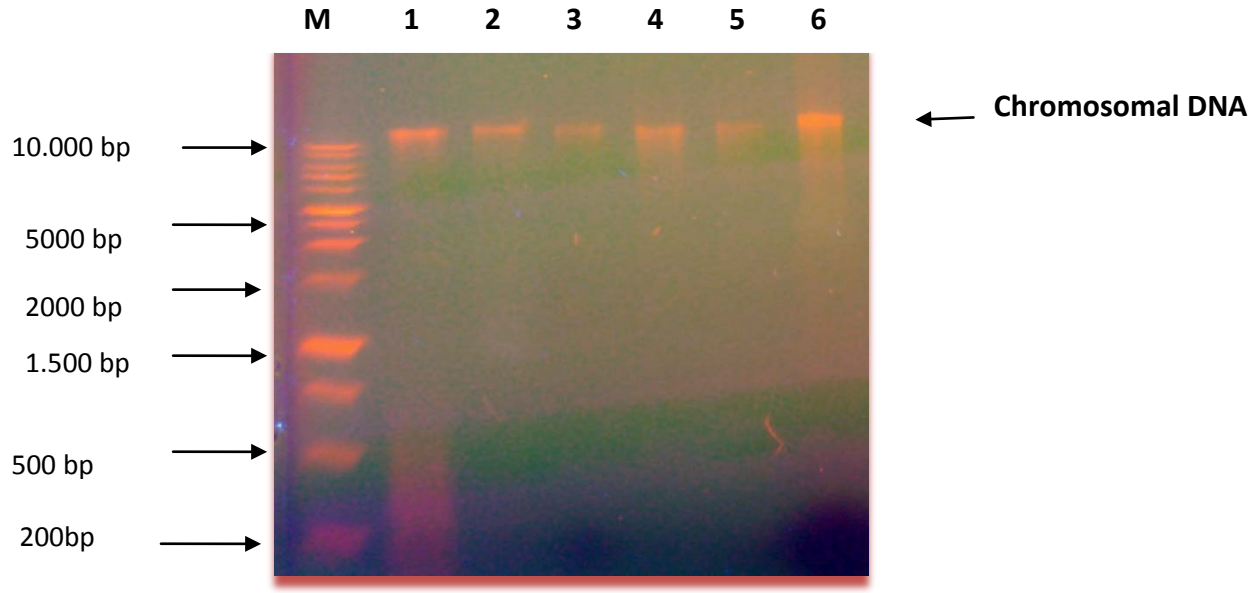


Figure (3-5): Gel electrophoreses of genomic DNA extracted by boiling method from the bacterial isolates. Electrophoreses was performed on agarose gel (0.7%) and run with 5 V/cm for 1- 3 hrs. Lane M is a (1 Kb) ladder, Lanes (1) DNA of *E. coli* extracted with ZnO (2) DNA of *E. coli* HB 101 (3) DNA of *E. coli* extracted with TiO_2 (4) DNA of *P. aeruginosa* (5) DNA of *p. aeruginosa* extracted with TiO_2 (6) DNA of *P. aeruginosa* extracted with ZnO.

- Alkali lysis method:

Results (table 3-4 and figure 3-6) explained improvement of quality of DNA. The purity of extracted DNA from *E. coli* HB 101 and *P. aeruginosa* was 1.6 and became 1.7 to 1.8 when DNA extracted with nanoparticles.

Results showed also that the concentration of DNA was highly increased when DNA isolated with ZnO nanoparticles from both bacteria. The concentration increased from 187 to 466 ng/µl for *E. coli* DNA and from 205 to 398 ng/µl for *P. aeruginosa* DNA. While the

concentration of DNA was not changed when isolated with TiO_2 nanoparticals for both bacteria (table 3-4).

Table (3-4): Concentration and purity of DNA extracted from *E. coli* and *P. aeruginosa* by using Alkali lysis method.

Extracted DNA From	DNA concentration ng\µl	Purity of DNA (260/280)
E. coli HB101	187	1.6
E. coli HB 101 with ZnO (0.4 mg/ml)	466	1.7
E. coli HB 101 with TiO_2 (0.2 mg/ml)	196	1.75
P. aeruginosa	205	1.6
P. aeruginosa with ZnO (0.4 mg/ml)	398	1.8
P. aeruginosa with TiO_2 (0.2 mg/ml)	201	1.7

From these result it can noticed that ZnO nanoparticles (0.4 mg/ml) had a good effect on DNA isolation and increased the yield of isolated DNA about 2 to 2.4 folds when extracted DNA by alkali lysis method in presence of ZnO.

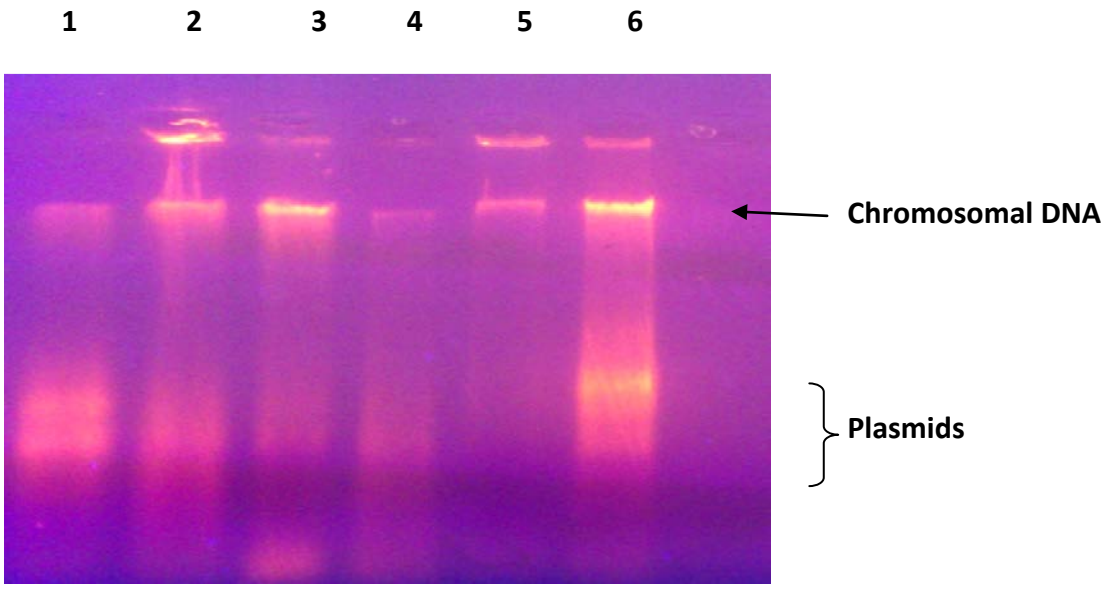


Figure (3-6): Gel electrophoreses of genomic DNA extracted by alkali lysis method from bacterial isolates. Electrophoreses was performed on agarose gel (0.7%) and run with 5 V/cm) for 1- 3 hrs. Lanes (1) DNA of *E. coli* HB101 extraced with TiO_2 (2) DNA of *E.coli* HB 101(3) DNA of *E .coli* extracted with ZnO (4) DNA of *P. aeruginosa* extracted with TiO_2 (5) DNA of *P. aeruginosa* (6) DNA of *P.aeruginosa* extracted with ZnO.

- Salting out method:

The results (table 3-5 and figure 3-7) declared high improvement of the quality of DNA. The purity of extracted DNA from *E. coli* HB 101 and *P. aeruginosa* was 1.6 and 1.5 and became 1.8 to 1.7 respectively, when DNA extracted with nanoparticles.

Results showed also that the concentration of DNA was highly increased when DNA isolated with ZnO nanoparticles from both bacteria. The concentration increased from 220 to 788 ng/µl for *E. coli* DNA and from 302 to 556 ng/µl for *P. aeruginosa* DNA; while there was little increase in DNA concentration for both bacteria when DNA isolated with TiO_2 nanoparticles (table 3-5).

Table (3-5): Concentration and purity of DNA extracted from *E. coli* and *P. aeruginosa* by using salting out method.

Extracted DNA From	DNA concentration ng\µl	Purity of DNA (260/280)
E. coli HB101	220	1.6
E. coli HB 101 with ZnO (0.4 mg/ml)	788	1.7
E. coli HB 101 with TiO_2 (0.2 mg/ml)	476	1.8
P. aeruginosa	302	1.5
P. aeruginosa with ZnO (0.4 mg/ml)	556	1.7
P. aeruginosa with TiO_2 (0.2 mg/ml)	466	1.7

The above results show that ZnO nanoparticles (0.4 mg/ml) had a good effect on DNA isolation and increased the yield of isolated DNA about 2 to 3.6 folds when the DNA was extracted by salting out method in the presence of ZnO nanoparticles. While presence of TiO_2 increased the yield of isolated DNA about 1.54 to 2.16.

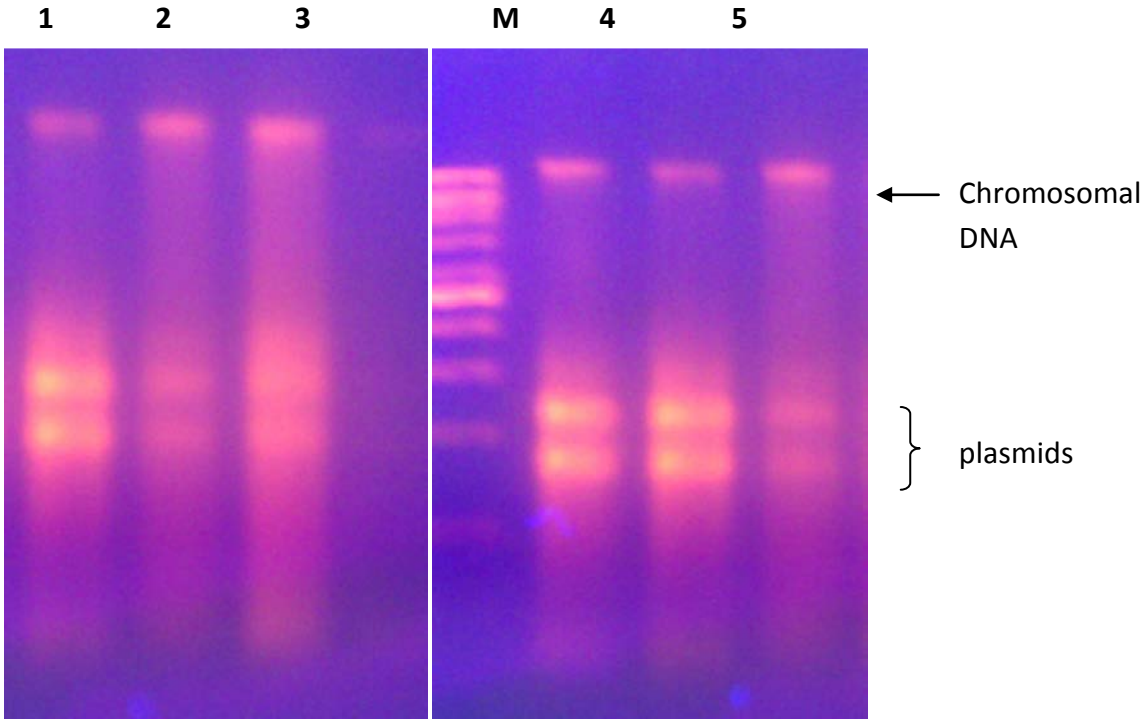


Figure (3-7)**:** Gel electrophoreses of genomic DNA extracted by salting out from the bacterial isolates. Electrophoreses was performed on agarose gel (0.7%) and run with 5 V/cm for 1- 3 hrs. Lane (M) is (1 Kb) ladder Lanes: (1) DNA of *P. aeruginosa* (2) DNA of *P. aeruginosa* extracted with TiO_2 (3) DNA of *P.aeruginosa* extracted with ZnO (4) DNA of *E. coli* HB 101 (5) DNA of *E. coli* extracted with TiO_2 (6) DNA of *E. coli* extracted with ZnO.

Results indicated that modified DNA extraction methods (Boiling, Alkali lysis and Salting out) in presence of ZnO nanoparticles gave a good enhancement of DNA quality and quantity of *E. coli* HB 101 and *P. aeruginosa*. While presence of TiO_2 lead to enhancement of DNA quality for both bacteria by all DNA extraction methods. However, TiO_2 caused enhancement of DNA quantity of both bacteria when used with salting out method only.

Bandyopadhyay *et al.* (2011) referred that some nanoparticles when used with commercial kit for DNA extraction, gave a high quantity of

genomic DNA. They explained also that extraction method with using nanoparticles can be performed in any laboratory without the requirement of sophisticated equipment. The procedure yields an ultrapure quality and at least equal quantity of DNA compared with the conventional (classic methods), purity also consuming with time of the method and least the chemicals that used in extraction.

From our results we can attribute the enhancement of DNA quantity and quality by using nanoparticles to increase the fragility of cell membrane and help to hydrolyzed it, and help to precipitate the proteins.

Bioneer used nanoparticles in mini plasmid kit, they referred to the fact that nanoparticles solution is effectively bound to the protein which aggregate and increase the total weight of the complex. It is also used as a lysate buffer in which it help the cell membrane to hydrolyses and exit all the contents [Bioneer/USA (WWW.bioneer.com)].

It was found that some nanoparticals like ZnO, TiO_2 and CuO, and silver oxide is tend to precipitate the proteins and obtained DNA with high purity (Yeates *et al.*, 1998; Bandyopadhyay *et al*., 2011).

Leng *et al.* (2006) referred that ZnO nanoparticles provide some protection against deoxyribonuclease (DNase) cleavage of DNA and may be inhibited the restriction enzymes.

3.4 Effect of nanoparticles on PCR amplification:

3.4.1 Extraction of DNA from *E. coli* HB 101 and *P. aeruginosa:*

Genomic DNA was extracted from *E. coli* HB 101 and *P. aeruginosa* using genomic DNA Qiagen kit (2.2.6.1). The extraction was done

according to the manufacturer's company after cultured on the Luria - Bertani broth. DNA was extracted to provide a PCR template for amplification. Results showed that the recorded range of DNA concentration was 95-202 ng/µl and the DNA purity was 1.6-1.8.

The obtained quantities and purity of DNA are fair enough for amplification by PCR. Higher amounts of DNA template increase the risk of generating of non-specific PCR products. Lower amounts of template reduce the accuracy of the amplification. Pure DNA preparation has expected of 1.8 which are based on the extinction coefficients of nucleic acids at 260 nm and 280 nm (Green and Sambrook, 2012).

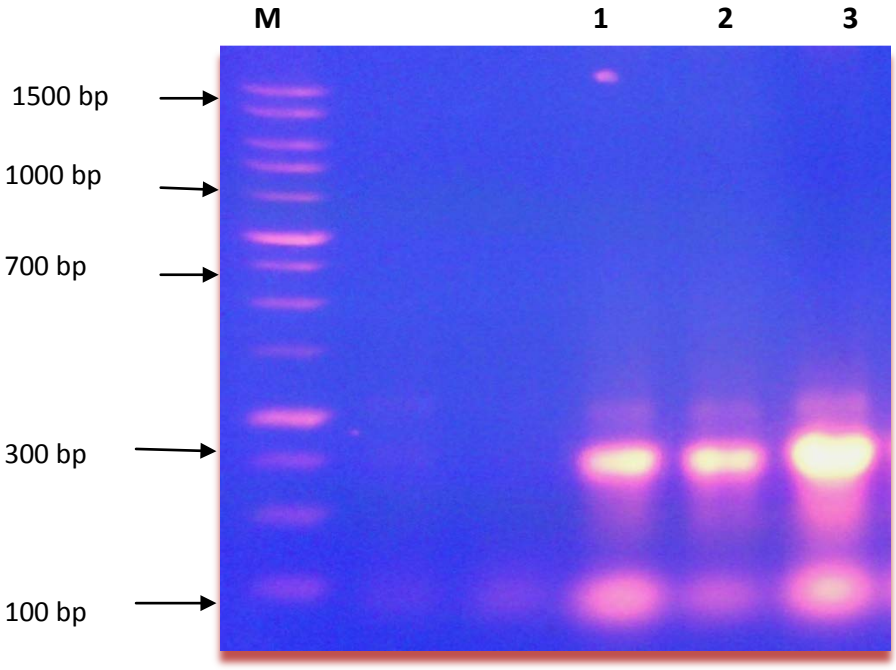
3.4.2 Amplification of *lac Z* and 16S rRNA genes:

Successful results (figure 3-8 and 3-9) were obtained with amplification lac Z and 16S rRNA genes, and electrophoresis of the amplified PCR products on 1.5 % agarose gels showed that DNA bands for the lac Z gene and 16S rRNA gene were appeared at the expected position. The amplified fragments were about 400 bp in size for lac Z gene and 300 bp for 16S rRNA gene, which are the same size obtained by Corinne (2004) and Spilker *et al.* (2004).

Results showed that there was an improvement of the DNA yield and purity when the amplification performed with nanoparticles. Different concentrations of nanoparticles were examined. It was found that 0.4 mg/ml of ZnO gave the best results. ZnO nanoparticles (0.4 mg/ml) were highly enhanced the PCR products for both genes (figure 3-8 and 3-9).

Determination of concentration and purity of amplified DNA (table 3-6) revealed also that the concentrations for both genes were highly increased, when amplification performed with ZnO nanoparticles. The

concentration increased from 255 to 488 ng/µl for lacZ gene of *E. coli* DNA and from 320 to 580 ng/µl for 16S rRNA of *P. aeruginosa.* The purity of these genes was improved also when using both nanoparticles. It was 1.7 and1.6 for lacZ gene and 16S r RNA and became 1.75- 1.8 and 1.7-1.8 respectively. This mean that the yield of lacZ gene of *E. coli* and 16S r RNA gene of *P. aeruginosa* was increased for about 1.9 and 1.8 folds respectively. While DNA concentration was not changed when genes amplified with TiO_2 nanoparticles for both bacteria (table 3-6).



Figur (3-8): Gel electrophoreses for amplification lac Z gene of *E .coli HB 101*. Electrophoreses was performed on 1.5 % agarose gel and run with 5V/cm for 1.5-2 hrs. Lane (M) is 100 bp ladder Lane: (1) Amplification without treatment. (2) Amplification with TiO_2 Np.(3) Amplification with ZnO Np.

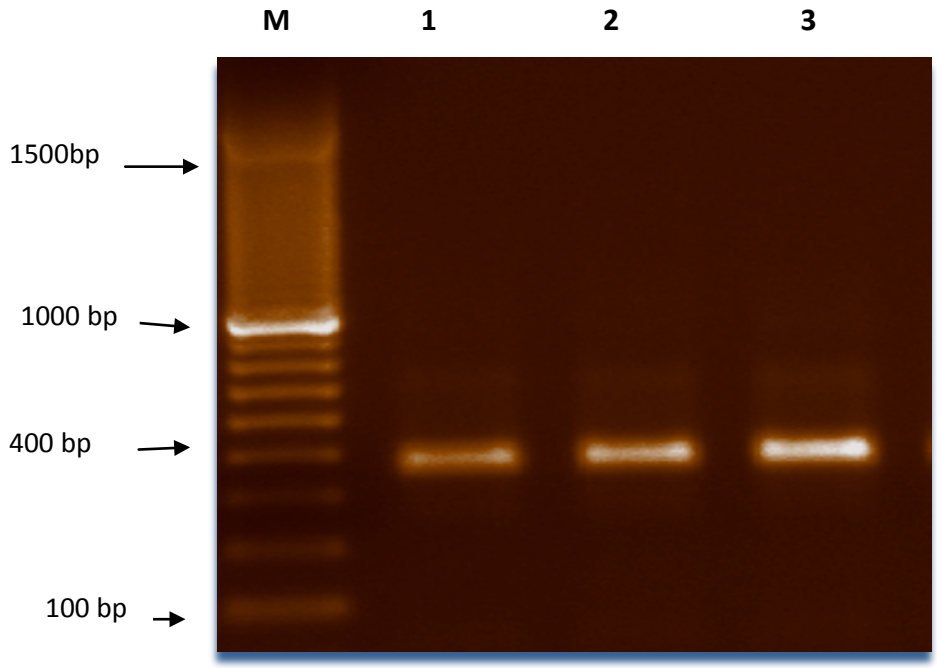


Figure (3-9): Gel electrophoreses for amplification 16S r RNA gene of *P. aeruginosa* Electrophoreses was performed on 1.5 % agarose gel and run with 5V/cm for 1.5-2 hrs. Lane (M) is 100 bp ladder Lane: (1) Amplification without treatment. (2) Amplification with TiO_2 Np. (3) Amplification with ZnO Np.

These results were obtained after many attempts and experiments, in which all PCR reaction conditions were manipulated (program time, number of cycling) with using nanoparticles. The optimum conditions were mentioned in (2.2.6.3). Different concentrations of nanoparticles ranging from 0.1 to 1 mg/ml were used, it was found that the best results were obtained when nanoparticles were used with concentration of 0.2 mg/ml for TiO_2 and 0.4 mg/ml for ZnO.

Table (3-6): Concentration and purity of amplified fragments of lacZ gene and 16S rRNA gene.

Amplification Sample	Concentration of amplified genes ng\µl	Purity (260/280) of amplified genes
lacZ gene of *E. coli* HB101	255	1.7
lacZ gene of *E. coli* HB101 with ZnO (0.4 mg/ml)	488	1.8
lacZ gene of *E. coli* HB101 with TiO_2 (0.2 mg/ml)	256	1.75
16S rRNA gene of *P. aeruginosa*	320	1.6
16S rRNA gene of *P. aeruginosa* with ZnO (0.4 mg/ml)	580	1.8
16S rRNA gene of *P. aeruginosa* with TiO_2 (0.2 mg/ml)	322	1.7

Results declared that there is 50 % obtained reduction on over all of the reaction time. This reduction was obtained by reducing the number of cycles (7-10 cycles) and the time periods of cycles (see item 2.2.6.3)

when using both nanoparticles. However ZnO nanoparticle – assisted PCR may be useful for profound reduction of the overall PCR reaction time and for enhanced amplification of DNA, but TiO_2 nanoparticles may be useful only for profound reduction of the overall PCR reaction time.

From these results it can be concluded that ZnO could be improve the specificity of PCR reaction by improving the specificity of the primers or enhancing thermal conductivity of the fluid, or may be it could inhibited the restriction enzymes.

Abdul Khalq *et al*. (2010) observed that optimal concentration (0.4 nM) of TiO_2 nanparticles (~ 25 nm) caused significant enhancement of PCR efficiency (7 fold increase in the amount of PCR product). They refered also that TiO_2 nanoparticles – assisted PCR is useful for reduction (as much as 50 %) in overall reaction time. They attributed that to a rapid increase in thermal conductivity of the fluid, caused by TiO_2 nanoparticlea and thereby contributed to the enhancement of PCR efficiency.

Nanoparticles seems to be attractive modulators of PCR, by virtue of their efficient heat transfer property. Gold nanoparticles have been shown to significantly improve both the specificity and sensitivity of PCR reaction (Li *et al*., 2005). Tetrapod – like ZnO nanoparticles, single walled carbon nanotube and carbon nanopowder were found to increase PCR efficiency and specificity (Cui *et al*., 2004).

Metal nanoparticles especially Au NPs and TiO_2 NPs were used the most frequently. The first report of NPs assisted PCR was focused on PCR specificity. It was reported that, in the presence of appropriate amount of NPs, it could obtain the target product (Pan *et al.*, 2007).

Li *et al.* (2005) stated that adding nanoparticles solution into PCR reagent was increased the PCR efficiency. The reaction time could be shortened too, without losing the amplification yield.

Li's group associated physical property of AuNPs (which it possess a good heat transfer efficiency in an aqueous solution) with their function of enhancing the PCR reaction. They proposed that heat conductivity of AuNPs plays the most important role in increasing the efficiency of the PCR thermal reaction by dramatically shortening the reaction time required (Li *et al*., 2005). There is another study which hold the same view point for their AgNPs-based PCR (Wang *et al.*, 2007).

Various nanomaterials open up new opportunities for improving PCR, which is the most important standard method in molecular biology. It is clear that a wide variety of optimized properties have been found with varied PCR systems, including GC-rich template amplification, long fragment DNA amplification, two rounds or multiple rounds PCR, RT-PCR, rapid PCR and so on. It is a positive outlook that most important properties of the PCR process can be improved using different nanomaterials, such as specificity, sensitivity, yields, reaction rate and so on. It is worth mentioning that Au Np. can retain higher specificity even at lower annealing temperatures. Furthermore, we realized hot start-like effect in gold nanoparticle-based PCR, which has promising applications in biological and biomedical studies (Dun *et al.,* 2011).

3.5 Effect of nanoparticles on Transformation:

In order to study the effect of nanoparticles on transformation, there have been certain attempts to transforme *E. coli* MM 294 (Rif^r, Am^s, Tc^s) with genomic DNA of *E.coli* HB101(Am^r, Tc^r, Rif^s) and *P. aeruginosa*

(Tc^r, Rif^s). Both bacteria were transformed successfully without using nanoparticles. Many transformation experiments were performed with using different concentrations of nanoparticles in order to increase transformation frequency.

Results showed that using ZnO (0.4 mg /ml) and TiO_2 (0.2 mg /ml) caused enhancement of *E. coli* MM294 transforments. Also other experiments were made in which the solution of $MgCl_2$ - $CaCl_2$ (80 mM, 20 mM) replaced with the nanoparticles solution (2.2.9) in which the chemical components were reduced. Transformation frequencies with *E. coli* HB 101 DNA were more increased when $MgCl_2$ - $CaCl_2$ solution was replaced with nanoparticles solution (figure 3-10). Transformation frequency was (1.7×10^{-3}) without using nanoparticles and (3.6×10^{-3}, 2.3×10^{-3}) with using ZnO and TiO_2 respectively.

Results showed also that transformation frequency of *E. coli* MM294 with DNA of *P. aeruginosa* was 1.2×10^{-3} and increased to 2.6×10^{-3} and 1.6×10^{-3} when using ZnO solution and TiO_2 solution respectively.

These results indicated that ZnO gave best improvement of transformation compared with TiO_2.

According to these results it can cocluded that using nanoparticles (ZnO or TiO_2) could increase transformation frequency of *E. coli* with genomic DNA of other Gram negative bacteria.

Some types of bacteria like *E. coli*, *Pseudomonas sp*., they don't have the ability to transform in which their cells are need to treats with some special factors to promote the competent cells, including chloride solution which increase the permeability of cell membrane (Tang *et al.*, 1994). Depending on our results, it can be concluded that ZnO and

TiO_2 nanoparticle solutions could be one of the factors that help to promote the competent cells by increasing their permeability through cell membrane.

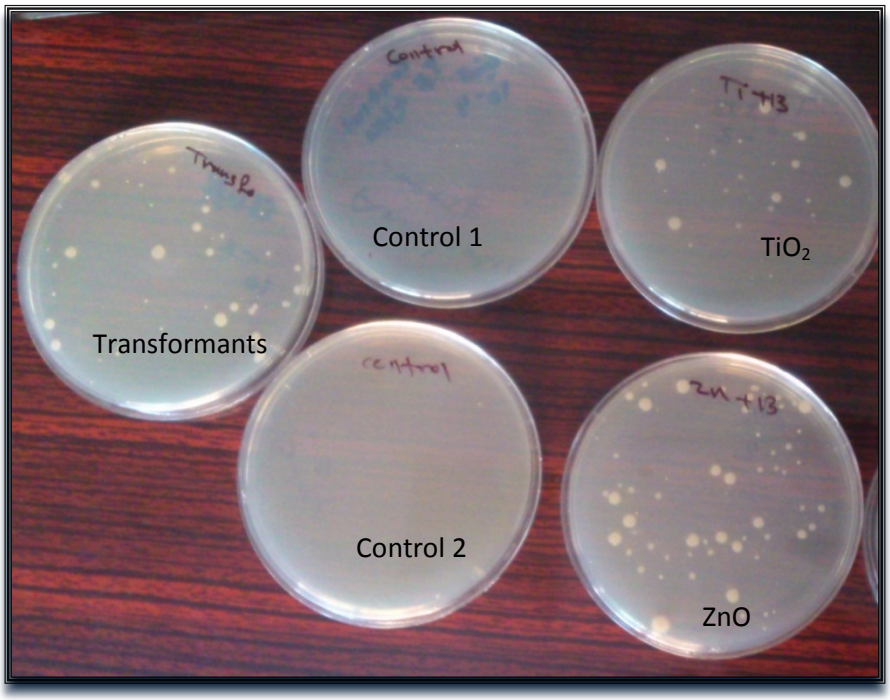


Figure (3-10): Effect of ZnO and TiO_2 NPs on transformation of *E. coli* MM294 with DNA of *E. coli* HB 101 plated on nutrient agar and incubated at 37C^o for 18 hrs.

Other factors may affect the transformation efficiency for example, Plasmid size, forms of DNA, geno type of cells, stages of growth of cell, methods of transformation, method of preparation of competent cells, the length of time of heat shock, temperature of heat shock, incubation time after heat shock, growth medium used, and various additives. These additives include adding calcium chloride, which help the bacterial cells to become a competent for easer transformation (Wilharm *et al*., 2010).

According to our Knowledge the present study is one of the limited studies about using nanoparticles in transformation of bacteria. In which transformation frequency was increased for about 1.3 to 2.1 folds.

One of these studies is based on using nanoparticles in Yoshida solution. This makes transformation rapid and simple and lead to enhanced transformation efficiency by approximately 50 % (Haidong *et al*., 2010).

3.6 Effect of nanoparticles on Conjugation:

In order to study the effect of nanoparticles on conjugation, attempts of conjugation were done between *E. coli* HB101(Am^r, Ctx^s) and *P. aeruginosa* (Am^s, Ctx^r), in presence of nanoparticles. Many experiments were performed, with using different concentrations of nanoparticles. Results indicated that there was no effect of any concentration of nanoparticals on conjugation, in which the frequency of conjugation remains the same with and without using nanoparticles which was about 1.7×10^{-2}. The same result was obtained when $MgSO_4$ solution was replaced with nanoparticles solution (2.2.10). In all these attempts, there were no effects by using ZnO and TiO_2 nanoparticles on conjugation freqency (figure 3-11).

Previous studies showed the possibility of conjugation between *E. coli*, as a recipient bacterium, and wild type *P. aeruginosa* due to the resemblance of their sex pili (Nasir *et al*., 2002). In addition, *E. coli* characterized with its rec^- and r^-, which means that entering plasmid do not suffer from restriction and recombination with the host chromosome, which leads to success of conjugation (Al-Amir, 1998).

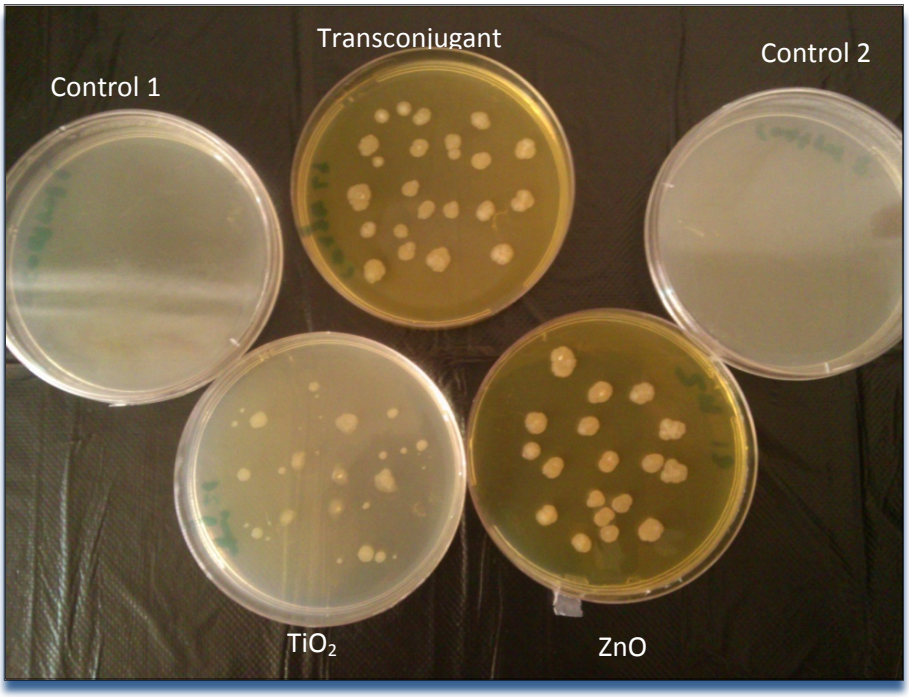


Figure (3-11): Effect of ZnO, and TiO_2 NPs on conjugation between *E. coli* HB 101 and *P. aeruginosa* plated on LB agar and incubated at 37C° for 18 hrs.

According to our knowledge this is the first attempt to study the effect of nanoparticles on conjugation. To make sure of this effect, it is recommended to carry out other attempt using other types of nanoparticles and bacterial species and strains.

Conclusions
And
Recommendations

Conclusions

- The nanoparticles (ZnO and TiO_2) used in this study were highly purified and their physical and chemical properties were not changed.

- Nanoparticles had antibacterial effect on Gram negative bacteria (*E. coli* HB 101, *P. aeruginos*a). The best antibacterial effect was at concentration 4 mg\ml and 3 mg/ml for TiO_2 and ZnO respeclively.

- An improvement in DNA quality was obtained when DNA extracted from Gram negative bacteria by extraction methods (boiling, alkali lysis and salting out methods) in presence of ZnO (0.4 mg/ml) or TiO_2 (0.2 mg/ml). The quality of DNA was improved also when ZnO used with all extraction methods, while TiO_2 coused improvement of DNA quantity when used with salting out methods only

  Improvements caused by these nanoparticles could be attributed to their role in cell membrane lysis and participating proteins.

- ZnO nanoparticles caused significant enhancement of PCR efficiency, increase the amount of PCR product for genomic DNA templates of both bacteria, and it is useful for profound reduction of the overall PCR reaction. While TiO_2 nanoparticles has no effect on the amount of PCR product, it may be useful only for profound reduction of the overall PCR reaction time.

  Improvement of PCR eficincy when using ZnO could be achieved by improving the specificity of the primers, enhancing thermal conductivity of the fluid, or inhibiting the restriction enzymes.

- Using nanoparticles (ZnO or TiO_2) in transformation experiments increased transformation frequency of *E. coli* with genomic DNA of other gram negative bacteria. And transformation frequency was more when using ZnO compaired with TiO_2.

- Transformation frequency increased more when $MgCl_2$-$CaCl_2$ solution was replaced with nanoparticle solution (reduced chemical components). Therfore these nanoparticles could promote the competence state of cells by increasing their permeability.

- There is no effect of nanoparticles on conjugation frequency between *E. coli* Hb101 and *P. aeruginosa.*

Recommendations

- Study the effect of using other types of nanoparticles on different molecular and genetic techniques, like cutting of DNA, real time PCR and curing of plasmid.

- Study the effect of nanoparticals on the growth and genetics of Gram positive bacteria.

- Further studies should be conducted to clarify the safety aspect of nanoparticles in vitro and in vivo.

- Immobilization of nanoparticles using different methods for applicable purposes.

- Other attempts to unify PCR conditions and investigate the effect of nanoparticles on anneling temperature.
- Other attempts to improve the transformation by investigating all factors which may affect this process.

- Other attempts are required, to make sure of the effect of nanoparticles on bacterial conjugation, using other types of nanoparticles and bacterial species.

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تم تضخيم جين *lacZ* لبكتريا *E. coli* وجين 16S rRNA لبكتيريا *P. aeruginosa* بوجود الدقائق النانوية. أظهرت النتائج ان اوكسيد الزنك بتركيز0.4 ملغم/مل عزز بشكل كبير نواتج التضخيم لكلا الجينين، كما تم تحسين نقاوة هذه النواتج عند استخدام كلا نوعي الدقائق النانويه.

أظهرت النتائج حصول انخفاض وصل الى حوالي 50 % من الوقت الكلي لتفاعل التضخيم باستخدام كلا نوعي الدقائق النانوية. وتبعا لهذه النتائج، يمكن الاستنتاج بان اوكسيد الزنك ربما يعمل على تحسين تخصص البواديء وتعزيز التوصيل الحراري للسوائل وتثبيط الانزيمات المقيدة.

من اجل دراسة تأثير الدقائق النانوية على التحول. تم تحويل بكتريا *E. coli* MM 294 بالدنا المجيني لكل من بكتريا *E. coli* HB 101 و *P. aeruginosa*. أوضحت النتائج ان 0.4 ملغم/مل من اوكسيد الزنك و 0.2 ملغم/مل من ثاني اوكسيد التيتانيوم قد عززا متحولات *E. coli* MM 294 . اشارت النتائج ايضا الى حصول زيادة اكثر بتكرار التحول عند استبدال محلول كلوريد المغنيسيوم – كلوريد الكالسيوم بمحلول الدقائق النانوية، والذي اختزل ايضا المكونات الكيميائية للمحلول.

كانت تكرارات التحول قد زادت حوالي 1.3 مرة و 2.1 مرة مع ثاني اوكسيد التيتانيوم و اوكسيد الزنك على التوالي . مما يشير الى ان اوكسيد الزنك اعطى افضل النتائج.

ولدراسة تأثير الدقائق النانوية على الاقتران. اجريت عدد من تجارب الاقتران ما بين *E. coli* HB 101 و *P. aeruginosa*، بوجود الدقائق النانوية. أشارت النتائج الى عدم وجود تأثير لاي من تراكيز الدقائق النانوية على الاقتران ، اذ بقيت اعداد المقترنات نفسها باستخدام او عدم استخدام الدقائق النانوية.

الخـلاصــة

هدفت هذه الدراسة الى اختبار تأثير استخدام دقائق اوكسيد الزنك (ZnO) ودقائق ثاني اوكسيد التيتانيوم (TiO_2) النانوية في بعض التقنيات الجزيئية لغرض تحسينها.

تم توصيف الدقائق النانوية المستخدمة في هذه الدراسة باستخدام جهاز المطياف للاشعة فوق البنفسجيه والمرئية والمجهر الالكتروني النافذ. أظهرت النتائج ان كلا الدقائق النانويه كانت عالية النقاوه وشكلها كروي ومتوسط قطرها حوالي 100 نانوميتر و 50 نانوميتر لثاني أوكسيد التيتانيوم واوكسيد الزنك على التوالي.

تم تحديد تأثير الدقائق النانوية (TiO_2 و ZnO) على نمو البكتريا. أوضحت النتائج ان لهذه الدقائق تأثيرمضاد للبكتريا وهي *Escherichia coli* HB1**0**1 و *Pseudomonas aeruginosa* (السالبة لصبغة غرام). وكان افضل تأثير مضاد للبكتريا هو التركيز 4 ملغم/مل لثاني اوكسيد التيتانيوم و 3 ملغم/مل لاوكسيد الزنك.

كان التركيز المثبط الادنى لأوكسيد الزنك على بكتريا *E. coli* HB 101 و *E. coli* MM 294 و *P. aeruginosa* هو 0.05، 0.05 و 0.5 ملغم/مل على التوالي. بينما كان التركيز المثبط الادنى لثاني اوكسيد التيتانيوم على هذه البكتريا هو 0.35 ملغم/مل.

أختبرت نوعية وكمية الدنا المعزول بطريقة الغليان والتحلل القاعدي والتمليح بوجود الدقائق النانويه. وجد ان اوكسيد الزنك عزز بشكل جيد نوعية وكمية الدنا المعزول من بكتريا *E. coli* و *P. aeruginosa* بينما ادى ثاني اوكسيد التيتانيوم الى تعزيز نوعية الدنا المعزول من كلا البكتريا بجميع طرق عزل الدنا. ومع ذلك فأن ثاني اوكسيد التيتانيوم أدى الى تعزيزكمية الدنا المعزول من كلا البكتريا عند استخدامه مع طريقة التمليح فقط.

الأهــداء

الى

روح اخي منذر..... رحمه الله

والدي ووالدتي ..... اداممما الله

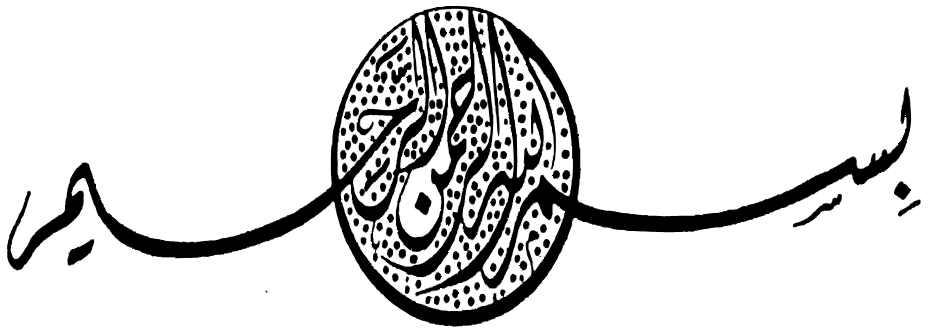
اخوتي واخواتي

زوجـــــي

أبنـائــي (لينة وعبد العزيز وتيم الله وجود).....محبة وعرفانا بجميل صبرهم

اهدي ثمره جهدي المتواضع هـذا..

منتهى



وأَنْزَلَ اللهُ عَلَيكَ الكِتابَ و الْحِكْمَةَ و عَلَمكَ ما لَمْ

تَكُنْ تَعلَمْ وَ كانَ فَضْلُ اللهِ عليكَ عَظيماً

صدق الله العظيم

سورة النساء (113)

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم
قسم التقانة الأحيائية

تأثير دقائق اوكسيد الزنك و ثاني اوكسيد التيتانيوم النانويه في استخلاص وتنقيه وتضخيم الدنا المجيني والتحول في بعض انواع البكتريا السالبة لصبغة كرام

اطروحة

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بإشراف

الاستاذ الدكتور	الاستاذ الدكتور
أ.د. ماجد حسين الجيلاوي	**أ.د. اياد محمد علي فاضل**
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