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Antibacterial Activity of Biosynthesis of Silver, Gold, and Iron Nanoparticles Against *Streptococcus mutans*

A Dissertation

Submitted to the Council of the College of Science at University of Babylon, in Partial Fulfillment of the Requirements for the Degree Doctorate of Philosophy of Sciences in Biology

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بِسْمِ اللَّـهِ الرَّحْمَـٰنِ الرَّحِيمِ

وَتَرَى ٱلْجِبَالَ تَحْسَبُهَا جَامِدَةً وَهِىَ
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سورة النمل (الآية 88)

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Dedication

If the Dedication expresses even part of the fulfillment, then the Dedication is due ...

To the Awaited Mahdi, son of the Prophet, and then to al-Sayyid Ali al-Sistani.

To my teachers and supervisor.

To my dear mother's soul, to my dear father.

To my dear brothers and sisters. To my dear wife and children.

To my loyal friends who stood with me in my scientific career.

To all science students who seek to obtain the highest levels of progress.

Hassanain, 2023

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Summary:

Streptococcus mutans is a facultative anaerobic, gram-positive coccus (round bacterium) commonly found in the human oral cavity and is a significant contributor to tooth decay. In the present study includes comparing the effect of three types of biosynthesized nano-materials, by using biological methods (synthesis of green nanomaterials) as antibacterial agents against *S. mutans* isolated from dental caries samples. The synthetic nano-materiales are silver nanoparticles Ag-NPs, gold nanoparticles Au-NPs, and iron nanoparticles Fe-NPs produced from *Bacillus clausii, Bacillus subtilis*, and oyster shells, respectively.

The characterizations of each type of nanoparticles were examined by X-Ray Diffraction XRD, Scanning Electron Microscope SEM, and Fourier Transform Infra-Red Spectrophotometric FTIR analysis, to ensure their shape, size, and purity, as well as to know that these synthesis of green nano-materials serve and give the desired purpose. The result of applied Scherrer Equation for the particle size measurements of silver Ag-NPs, gold Au-NPs, and iron Fe-NPs was equal to: 43.18 ,10.69, and 69.37 n.m, respectively.

Hundred samples of dental caries were collected by transport media (invasive sterile collection), from patients that they visited the Educational Clinics of the College of Dentistry / University of Babylon from April \ 2022 to August \ 2022). Out of these specimens 64 are found to be negative, while 36 specimens were positive indicated to the presence of *S. mutans*.

Subsequently, isolation and identification of *S. mutans* were carried out using various methods represented by gram stain followed by morphological examination used microscopic, and the confirmation of identification of *S. mutans* was achieved through the technique of

polymerase chain reaction PCR screening via amplification of specific gene (*Sm479* gene sequence).

The detection of biofilm performed using the Enzyme Linked Immune Sorbent Assay ELISA technique by microtitter plate, the result involved 14 isolates non-adherence, 61 weak-adherence, 16 moderate-adherence, and 4 strong-adherence), about (14.73%, 64.21%, 16.84%, and 4.21%) respectively.

Sensitivity and resistance of *S. mutans* to 17 classes of antibiotics through the "Antibiotic Sensitivity Test AST". In the results of this study the *36 S. mutans* isolates showed resistance in the range (22.22%-100), and moderate for about (non-36.11%), while the sensitive percentages were (non-41.66%).

Anti-bacterial activity of nano-materials on *S. mutans* (anti-biofilm production and agar disc diffusion test) performed, (AuNPs, AgNPs and FeNPs) recorded high effective as an antibacterial against *S. mutans*

With the application of AuNPs the measurements of inhibition zones ranging between 10-21mm were recorded. At a concentration of (400 mg/ml) higher than other concentrations, as they range between zone of inhibitions between 18-21mm, followed by the concentration of 200 mg/ml it was about 16-18mm inhibition zones. The concentration of 100 mg/ml was shown about 13-16mm inhibition zones, while the concentration of 50 mg/ml was appeared less measurements of inhibition zones, about 10-13mm.

With the application of AgNPs the measurements of inhibition zones ranging between 13- 20 mm were recorded. At a concentration of 400 mg/ml higher than other concentrations, as they range between zone of inhibitions between 17-20mm, followed by the concentration of 200 mg/ml it was about 16-18mm inhibition zones. The

concentration of 100 mg/ml was shown about 13-15mm inhibition zones, while the concentration of 50 mg/ml was appeared less measurements of inhibition zones, about 13-14mm. When FeNPs applied against *S. mutans*, the inhibition zone measurements were 16-20mm, 14-17mm, 12-14mm, and 10-13mm with the concentration of 400 mg/ml , 200 mg/ml, 100 mg/ml, and 50 mg/ml respectively.

Minimum Inhibitory Concentration MIC tests of AuNPs, FeNPs, and AgNPs against *S. mutans* were performed and recorded 100, 125, and 75 mg/ml, respectively. While the Minimum Bactericidal Concentration MBC of AuNPs, FeNPs, and AgNPs against *S. mutans* 125, 125, and 100 mg/ml respectively.

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List of Abbreviations

Abbreviation	Abbreviation
FeNPs	iron nanoparticles
AuNPs	gold nanoparticles
AgNPs	silver nanoparticles
nm	nanometer
SEM	Scanning Electron Microscope
XRD	X-Ray Diffraction
FTIR	Fourier Transform Infrared Spectroscopy
ROS	reactive oxygen species

IONP	**Iron oxide nanoparticles**
µg/ml	**Microgram\mil letter**
DPPH	**2,2-Diphenyl-1-picrylhydrazyl**
NADPH	**Nicotinamide Adenine Dinucleotide Phosphate**
ESR	**electron spin resonance**
PCD	**programmed cell death**
EPS	**extracellular polysaccharides**
GTF	**glucosyltransferases**
FTF	**fructosyltransferases**
HCl	**Hydrochloric acid**
PBS	**Phosphate buffer saline**
AuCl3	**Gold Chloride**
NaOH	**Sodium hydroxide**
(TE) buffer	**Tris-EDTA buffer**
BHIB	**Brain heart infusion broth**
BHIA	**Brain heart infusion agar**
CFM 5 mcg	**Cefixime**
E10	**Erythromycin**
CAZ 10	**Ceftazidime**
PIT	**Piperacillin/tazobactam**
AZM-15	**Azithromycin**
CRO 10	**Ceftriaxone**
RA 5	**Rifampicin**
DA 10	**Doxycycline**

LEV 15	**Levofloxacin**
CEP 10	**Cephalosporin**
FEP 10	**Fluorinated ethylene propylene**
SXT 25	**sulfamethoxazole**
MEM 10	**Meropenem**
NA 30	**Nalidixic acid**
IPM 10	**Imipenem**
TE 10	**Tetracyclin**
CIP 10	**Ciprofloxacin**
CFM 5 mcg	**Cefixime**
E10	**Erythromycin**
CAZ 10	**Ceftazidime**
PIT	**Piperacillin/tazobactam**
AZM-15	**Azithromycin**
CRO 10	**Ceftriaxone**
RA 5	**Rifampicin**
DA 10	**Doxycycline**
LEV 15	**Levofloxacin**
CEP 10	**Cephalosporin**
F (Sense)	**Forward (Sense)**
R (antisense)	**Reverse (antisense)**
AST	**Antibiotic Sensitivity Test**
MHA	**Muller Hinton Agar**
MIC	**Minimum Inhibitory Concentration**

MBC	**Minimum Bactericidal Concentration**

Chapter One

Introduction

1. Introduction

1. 1. Introduction

Dental caries is one of the most common chronic dental diseases worldwide. Dental Caries, otherwise known as tooth decay. If it is not diagnosed and treated in time, it will cause tooth loss disquiet in the act of chewing and speaking affecting the burden and beauty of a person (Wafiullah Amini, *et al.,* 2022; Yadav K, Prakash S, 2017). Dental caries is a multifactorial disease that involves microbial, behavioral, genetic, and environmental factors. Although these factors are important in caries development, the role of microbial factors cannot be ignored. (Zhang, J.S. *et al.,* 2022).

Oral cavity infection can occur due to the activity of germs that are already present in the mouth naturally, and these injuries include tooth decay, abscesses, periodontal infections, and gingivitis (Holt, *et al.*, 2000). *Streptococcus mutans*, a major etiological agent for caries, in association with the *lactobacillus* and *Actinomycetes* in dental diseases (Dinis, M. *et al.,* 2022). *Streptococcus mutans* is the main causative agent in the development of dental caries, among them, *Streptococcus mutans* and *Streptococcus sobrinus* that are isolated from human teeth, are considered to be the most important species associated with dental caries (Marsh, P.D. 2003).

Biofilm, also known as plaque, initially forms as a result of the action of virulence factors that bacteria use during the caries stages. The production of nanoparticles using biosynthesis has a well-defined size that can be controlled , and the probability of pollution is low in this synthesis process, and it is a very easy and wide-ranging method (Rónavári, A. *et al.,* 2017). Because nanoparticles have anti-biofilm

activities, and can penetrate them more effectively than free drug molecules, they can be applied in the field of bio-sensitivity and medicine (R. Y. *et al.*, 2013 ; R. P. Dhavale, 2021).

There is a great interest in gold nanoparticles among the rest of the various other metallic nanoparticles due to their unique properties that they possess, including nano size, and the fact that they do not cause cytotoxicity (especially when created by biological methods), they are created in relatively simple ways, and accurate targeting (Fu LH. *et al.*, 2017). Moreover, these gold nanoparticles are considered an effective therapeutic means to eliminate multidrug-resistant pathogens by binding to bacterial DNA, which leads to preventing DNA uncoupling during the transcription process through its binding to bacterial DNA (Arafa MG. *et al.*, 2018).

There are multiple applications of silver nanoparticles in killing different types of bacteria, such as adding them to socks to kill bacteria associated with foot odor, and using them in washing machines due to their antimicrobial activity (Vigneshwaran N. *et al.*, 2007; Chen X. *et al.*, 2008).

The antimicrobial activity of iron nanoparticles be demonistrated by the accumulation of particles in the cytoplasmic region, where while the smaller the nanoparticle better the penetration and accumulation capacity within the cell wall of the bacteria must be, in order to cause the rupture of the membrane through which the fugues of cellular material occurs and finally the death of the bacteria (C. Devathaa *et al.*, 2018).

The present work aims to study antibacterial activity of AgNPs, AuNPs, and FeNPs against *Streptococcus mutans* isolated from dental caries, by the following objectives:

- Isolation and diagnosis of *S. mutans* from dental caries on the modified Mitis Salivarius Agar Base.
- Biosynthesis of silver nanoparticles AgNPs by *Bacillus clausii*. Biosynthesis of gold nanoparticles AuNPs by *Bacillus subtilis*. Biosynthesis of iron nanoparticles FeNPs by Oyster shell.
- Characterization of biosynthesized AgNPs, AuNPs, and FeNPs by XRD, SEM and FTIR .
- Detection the Antibiotics Susceptibility Test (AST) of AgNPs, AuNPs, and FeNPs against *S. mutans*.
- Anti-bacterial activity of AgNPs, AuNPs, and FeNPs against *S. mutans*.
- Detection of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of nanoparticle.

Chapter Two
Literature review

2. Literature Review

2.1. Dental Caries

Dental caries is a microbial-mediated oral disease initiated by the presence of stagnant plaque biofilms, where pathogens such as *S. mutans* metabolize dietary sugars and produce acids. This results in dysbiosis, which sustains *S. mutans* biofilm growth, creating acidic microenvironments that demineralise the dental hard tissues, leading to dental caries (Vijayakumar *et al*., 2021).

Oral cavity microbiota reaches a certain level of stability in adulthood; however, its state can be easily disrupted, for example, by insufficient oral hygiene and tooth loss (Lemos, *et al.,* 2019, Stuz˙ycka, *et al.,* 2019).

Acid production and the glucan-rich matrix in *S. mutans* biofilms enable the spatial orientation of other cariogenic microbes constituting an elaborate microbial network leading to the development of caries (D. Kim *et al*., 2020). In addition, multiple virulence factors, including acid and stress tolerance, cell persistence, genetic competence and bacteriocin production collectively contribute to the progression of dental caries (Shanmugam *et al*., 2020).

Common aspects of the lifestyle, including antibiotics, high-fat diets, alterations in saliva and even actions and experiences can persistently change commensal microbial communities (David *et al*., 2014). Some microorganisms that colonize the oral cavity can form a dental plaque. Plaque formation can lead to two of the most common oral diseases; dental caries and periodontal diseases. The absence of oral hygiene, one of the essential reasons, causes the plaque to accumulate (Abo Bakr *et al*., 2021).

Tooth decay is expressed as a chronic local inflammation that changes the structure of the teeth as a result of the loss of chemicals resulting from metabolic activity in the body, as it occurs due to the formation of dental biofilms on the surface of the tooth (Chen X. *et al.,* 2008).

The change that occurs to the tooth as a result of decay is reversible and can be stopped in its early stages. There are several factors that are the cause of dental caries, including saliva, excessive dietary consumption of sugar, and exposure to fluoride, which affects the dynamic balance between demineralization processes on the one hand and remineralization on the other hand (Selwitz. *et al.,* 2007).

The pathogens are also multifactorial, with no single causal mechanism also (Featherstone. *et al.,* 2004), but the role of microbes here plays a key role in this aspect. Oral health or disease between these stages are strongly correlated, as caries experience in primary teeth often influences the caries outcome in permanent dentition (Saethre-Sundli. *et al.,* 2022).

There are various factors affecting the pattern of spread and severity of tooth decay, including gender, age, race, socio-economic status, as well as geographical location, dietary habits and oral hygiene, whether in the same country or in different parts of the world (Fejerskov., 2004; Petersen. *et al.,* 2005).

There has been much interest in the use of antibacterial agents (antibiotics) whose purpose is to treat tooth decay, and to prevent it greatly in the world (Sweeney. *et al.,* 2004).

The most common carcinogenic and predisposing of oral *streptococci*, is *S. mutans*. It has adapted to survive in the oral environment, and to metabolize a wide range of carbohydrates by the non-oxidative pathway

(Ajdic. *et al.*, 2002). Caries occurs as a result of bacterial secretions in the mouth, which leads to a gradual local disintegration of the dental tissues. Polymerase chain reaction amplification methods have been widely used in order to distinguish this type of bacteria and reveal its specificity, and this method is considered the reliable diagnostic tool for examining bacterial isolates (Nakano, *et al.*, 2006).

The pathogenic *S. mutans* possesses a distinctive feature, which is adhesion, which enables it to establish colonization on the surface of the teeth in preparation for the occurrence of tooth decay (Banas. 2004), and the formation of an obvious biofilm on it (Yoshida and Kuramitsu, 2002).

2.1.1.Types of Dental Caries

2.1.1.1. Primary caries

This type of decay occurs on the surface of the tooth, and the lesion begins to appear just below the contact area between the teeth. This is a primary phenomenon of decay and is localized, leading to the occurrence of a pit or crack on the surfaces. In this type of caries in the enamel layer, a process known as 3-dimential works to remove the minerals below the surface and spread along the prisms of the enamel layer (Yadav, 2016), as shown in figure (2.1).

2.1.1.2. Secondary caries

This type of caries represents the second stage of dental caries, and it is the lesion that occurs on the margin of tooth restoration, and it is characterized by that it begins next to the wall of the cavity, transcending to the layer that is below the enamel layer, and is the result of microscopic penetration. However, clinical and microbiological studies

indicate that this leakage does not lead to active metal removal below the restoration (Yadav, 2016), as shown in figure (2.1).

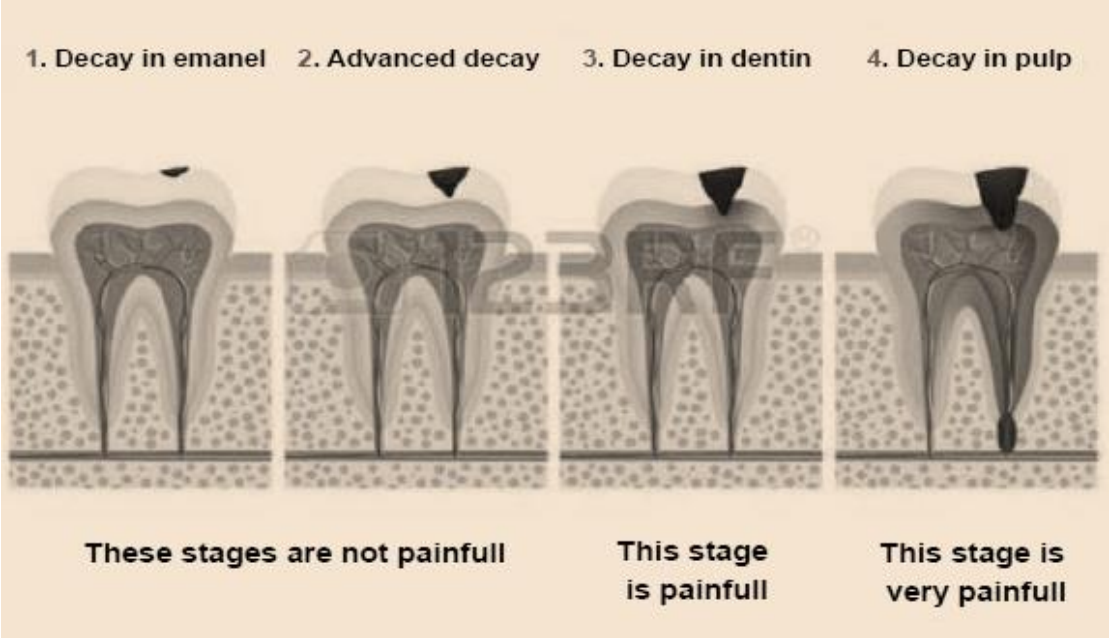


Figure (2.1): Schematic diagram for dental caries stages (primary and secondary stages). (Jennifer, and Jill, 2020).

2.2 Causes of Dental Caries

Dental caries is an infectious illness that involves the gradual breakdown of hard tooth tissue through a process of demineralization. The development of this disease is influenced by multiple elements that interact and work together, specifically the host (teeth and saliva), agents (microorganisms), substrate (diet containing sugar), and time. Dental plaque, also known as a biofilm, is formed on the tooth surface through the combination of saliva, substrate, and bacteria (Chismirina *et al.*, 2021).

2.2.1 Factors related to the host (tooth and saliva)

The tooth structure is crucial, as certain parts of the same tooth are more susceptible to infection than others, possibly due to variations in mineral content, particularly fluoride (Men *et al.*, 2016). A correlation has

been shown between the salivary parameters, including calcium, inorganic salivary phosphate, salivary pH, and dental caries (Nicolae *et al.*, 2016).

2.2.2 Microbial Agents

Mutans Streptococci (MS) are frequently linked to being significant cariogenic bacteria. *Streptococcus mutans* is found among the collection of microorganisms in the mouth known as oral flora. It has been proven to be a primary factor in the development of dental caries due to its ability to convert fermentable carbohydrates into organic acids through metabolic processes. These acids have the potential to lower the pH level, resulting in an increase in the solubility of enamel, which can lead to the development of dental caries (Al-Shami *et al.*, 2019).

2.2.3 Substrate

Is a material or substance on which a biological reaction or process takes place. The substrate plays a crucial role in the development of dental caries since it provides the necessary nutrients for bacteria to undergo fermentation via glycolysis, resulting in the production of acidic byproducts. The fermentation process generates acid that rapidly lowers the plaque pH below the crucial threshold within a time frame of 1-3 minutes. Tooth demineralization occurs when minerals in tooth enamel dissolve due to a gradual fall in pH. Continuous demineralization leads to the development of white patches on the tooth surface, ultimately resulting in the formation of cavities. The medical term for this ailment is dental caries (Yadav *et al.*, 2020).

2.3 Streptococci

Streptococci constitute a significant percentage of the oral flora that resides in the mouth. Approximately 25% of the cultivable flora found in gingival and supragingival plaque, and 50% of the isolates from the tongue and saliva, consist of Streptococci. These bacteria are transferred from mother to child in a vertical manner. Infective endocarditis caused by these bacteria typically occurs when they enter the bloodstream during intraoral surgical operations, such as tooth extraction, and sometimes even during tooth brushing (Health *et al.*, 2014).

Oral Streptococci are a significant population in the mouth, with many species inhabiting different areas of the oral cavity. The oral group is occasionally referred to as the viridans Streptococci due to the erythrocytes' incomplete clearance around the colony. According to (Moreira *et al.*, 2015), the oral Streptococci bacteria are currently classified into four species groups: Mutans, Salivarius, Anginosus, and Mitis group.

Streptococci are Gram-positive bacteria characterised by their spherical or ovoid cells, which have a diameter ranging from 0.5 to 2.0 μm. They typically develop in pairs or chains. These microorganisms lack the enzyme catalase and are typically able to survive in both aerobic and anaerobic conditions. They thrive best in a nutrient-rich environment and sometimes need 5% carbon dioxide for optimal growth. The bacteria exhibit an optimal temperature of approximately 37 ºC, while the lowest and maximum temperature can vary across the genus (Hulting, 2016).

The organisms are categorised into three distinct groups based on the kind of hemolysis observed on blood agar: beta-hemolytic (resulting in complete destruction of red blood cells), α-hemolysis (characterised by

green hemolysis), and gamma-hemolytic (where no hemolysis occurs). Beta-hemolytic Streptococci can be classified into two groups: group A Streptococci, which is also known as Streptococcus pyogenes, and group B Streptococci, which is also known as Streptococcus agalactiae (Kanwal and Vaitla, 2022).

2.4. *Streptococcus mutans*

The major Gram-positive, non-haemolytic (G-haemolytic) species of oral Streptococcal bacteraemia include *Streptococcus mutans*. *S. mutans* is a bacterium that is Gram-positive, non-motile, non-spore producing, and catalase negative. It is a facultative anaerobe and grows best at a temperature of 37°C. Under microscopic examination, it is usually found in pairs or chains (El Sherbiny, 2014).

S. mutans was initially documented in 1924 by J. Kilian Clarke, who observed minute, oval-shaped coccobacilli arranged in chains within deep dentin caries lesions. He proposed that these germs were genetic variants of Streptococci and designated them as *S. mutans* (Grönroos, 2000). Nevertheless, *S. mutans* garnered significant interest from the scientific community in the late 1950s, and by the mid-1960s, it was acknowledged as a primary causative agent in dental caries (Lemos *et al*., 2013).

S. mutans strains exhibit fast acid generation while fermenting glucose, lactose, raffinose, mannitol, inulin, and salicin. The tight adhesion of S. mutans colonies to the tooth surface is considered highly significant (Gharajalar and Hassanzade, 2017).

S. mutans bacteria are Gram-positive cocci that are facultatively anaerobic and belong to the group of lactic acid-producing bacteria. The breakdown of carbohydrates and the formation of lactic acids (acidogenicity) are crucial virulence features of *S. mutans*, leading to the

development of tooth decay. Furthermore, *S. mutans* bacteria possess acidouricity, which enables them to counteract the detrimental acidic conditions when subjected to pH levels that are not deadly (Abo Bakr *et al.*, 2021).

S. mutans plays a crucial role in the development of harmful dental biofilms, primarily because it may produce extracellular polysaccharides such water-insoluble glucans or fructans through the activity of GTFs and FTF (Aqawi *et al.*, 2021). *S. mutans* also synthesises many Gbp, which are believed to enhance attachment to matrix glucans and influence the overall structure of the biofilm (Jakubovics *et al.*, 2021). *S. mutans* naturally resides in the oral cavity of humans. On sheep blood, it primarily exhibits α or γ-hemolysis, although there are a few strains that display β-hemolysis (Zhou and Li, 2021).

2.4.1. Virulence Factors of *Streptococcus mutans*

Virulence factors of *S. mutans* help to protect the bacteria against possible host defenses and maintain its ecological niche in the oral cavity, while contributing to its ability to cause host damage (Health *et al*., 2014).

The most important virulence features associated with cariogenicity and distinguished of *S. mutans* strains from the other oral Streptococci isolated from the human oral cavity are: (1) capability of the bacteria to produce enormous amounts of organic acids from carbohydrate metabolism; (2) the potency of the bacteria to reside at lower pH (aciduricity); and (3) the excellency to synthesis extracellular glucan homopolymers using sucrose and all these characters perform the acts like early adherence, colonization and buildup of biofilms on tooth surfaces (Lemos *et al*., 2013).

Mutans streptococci and lactobacilli are strong acid producers and hence cause an acidic environment creating the risk for cavities (Tanzer, J.M. *et al.,* 2001). Usually, the appearance of *S. mutans* in the tooth cavities is followed by caries after 6-24 months (Mayooran, *et al.,* 2000).

The acidogenic *S. mutans* and *S. sobrinus* are able to form extracellular polysaccharides (EPS) in the presence of sucrose (Zero, 2004), but also from fructose and glucose. The EPS are long-chained and high molecular mass polymers (Zisu and Shah, 3003).

The energy rich glycosidic bond between the glucose and fructose moieties supplies the free energy needed for the synthesis of EPS. Glucose homopolysaccharides are called glucans while fructose homopolysaccharides are called fructans (Monsan, *et al.,* 2001).

The first property is completely dependent on the synthesis of water-soluble glycans that come from sucrose (a disaccharide). Second, I possess the ability to develop more acid tolerance. Which leads to the third characteristic that displays a production of lactic acid from dietary sugars, as shown in figure (2.2).

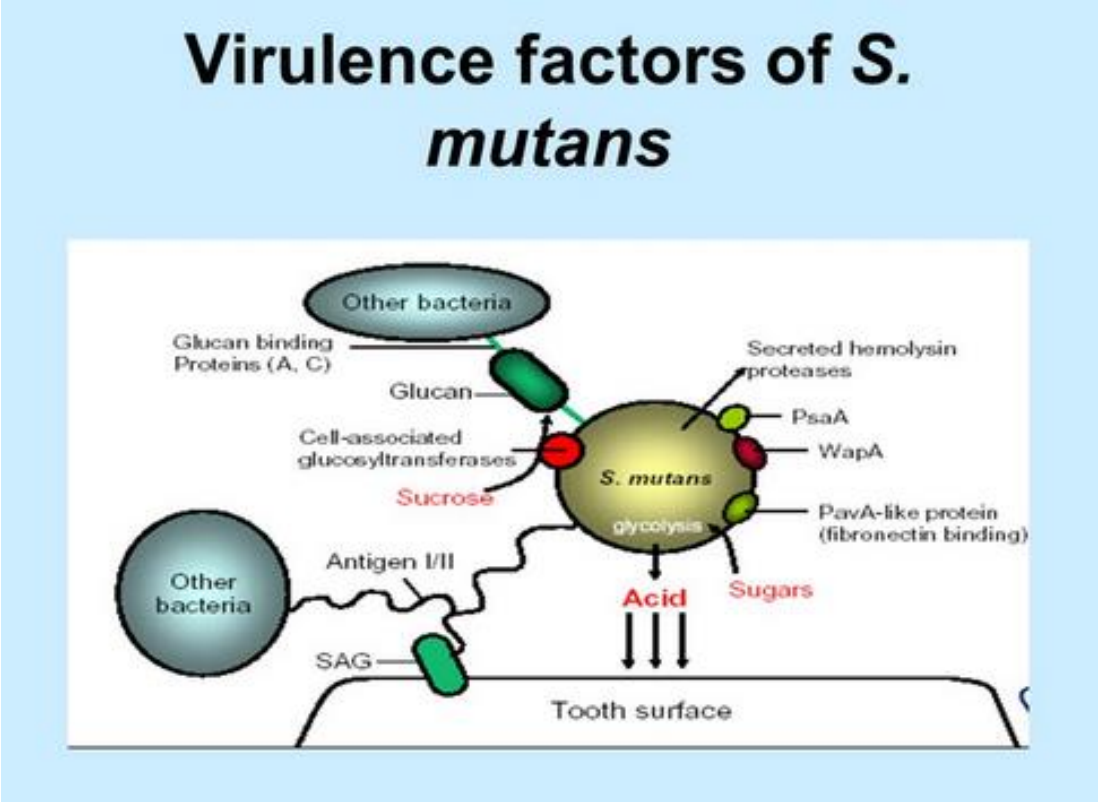


Figure (2.2): There are three very crucial and significant virulence factors which are associated with the carcinogenicity of *S. mutans*. (Krzyściak, W. *et al.*, 2014).

2.4.1.1 Bacterial Adhesion

The formation of dental plaque is a result of intricate and multi-step procedures. The initial stage involves the development of the acquired pellicle. The enamel surface, which is encompassed by the hydration layer, has a negative charge because to its elevated concentration of phosphate groups. Cations, such as calcium ions, adhere to the negatively charged enamel surface, ultimately altering it to a positive charge. Saliva contains acidic proteins, specifically phosphoproteins and sulphate glycoproteins, that possess a negative charge. Acidic proteins adhere to the enamel surface by binding to calcium ions, resulting in the formation of acquired pellicle. The calcium ion serves as a mediator between the negative charge on the enamel surface and the negative charge of acidic

proteins. Oral bacteria adhere to the acquired pellicle on the surface of the tooth. The initial stage of bacterial capture involves a reversible attachment that occurs due to either an ionic connection or van der Waals interaction (Huang *et al.,* 2013).

2.4.1.2 Extracellular and Intracellular Polysaccharides Formation

The matrix of dental plaque is created following the colonisation of oral bacteria. The plaque matrix can be generated in the absence of nourishment. The extracellular polysaccharides contribute to the thickening and hardening of dental plaque, reducing its oxygen permeability. They also play a crucial role in the maturation of dental plaque (Shemesh *et al.,* 2007).

S. mutans has the ability to synthesize intracellular polysaccharides (IPS), which is another virulence feature they exhibit. *S. mutans* metabolizes the stored carbohydrates to generate acid, which increases carcinogenicity by sustaining an acidic pH (<5.5) in the surroundings (Bao *et al.,* 2015).

2.4.1.3 Biofilm Formation

The dental plaque formed in oral cavity is a mixed population of biofilms made by various bacteria combining themselves. Oral Streptococci species such as *Streptococcus sanguinis*, *S. mutans*, *Streptococcus gordonii*, *Streptococcus mitis*, *and Streptococcus oralis* play the important role in the formation of supragingival plaque and dental caries. *Actinomyces* species such as *Actinomyces viscosus* are closely related to the development of dental plaque and dental caries on the root surface (Larsen and Fiehn, 2017).

These bacteria combine with the protein matrix formed by the oral bacteria and the extracellular polysaccharides such as glucan and fructan to form the dental plaque on the tooth surface. The *relA* gene carried by *S. mutans* encodes RelA, which is known to regulate the formation of biofilm and to contribute to quorum-sensing (Lemos *et al*., 2004).

Mutual and cooperative interactions in biofilms are essential for ecological balance, but species often compete for space and nutrients. The dominant growth of a few competitive species such as *S. mutans* often leads to disease pathogenesis. *S. sanguinis*, which coexists in the biofilm, keeps *S. mutans* in check by producing harmful H2O2 (Kreth J. *et al.*, 2005). *S. mutans* counteracts with the expression of Glutathione biosynthesis bifunctional protein GshAB, a recently discovered gene involved in detoxifying H2O2 (X Zheng. *et al.*, 2013).

The specific mechanism is still under research. *S. mutans* also produce bacteriocins, also known as bacterial toxins. For example, *S. mutans* UA159 can synthesize mutacin V and IV (MS Hossain, and I Biswas. 2011). In a multispecies community, mutacin gives a competitive advantage by excluding other organisms, but the high cost of mutacin synthesis can slow down growth. Thus, in carbohydrate-rich environments, *S. mutans* channel energy towards growth instead of microbial antagonism, as shown in figure (2.3) (J Kreth. *et al.*, 2005).

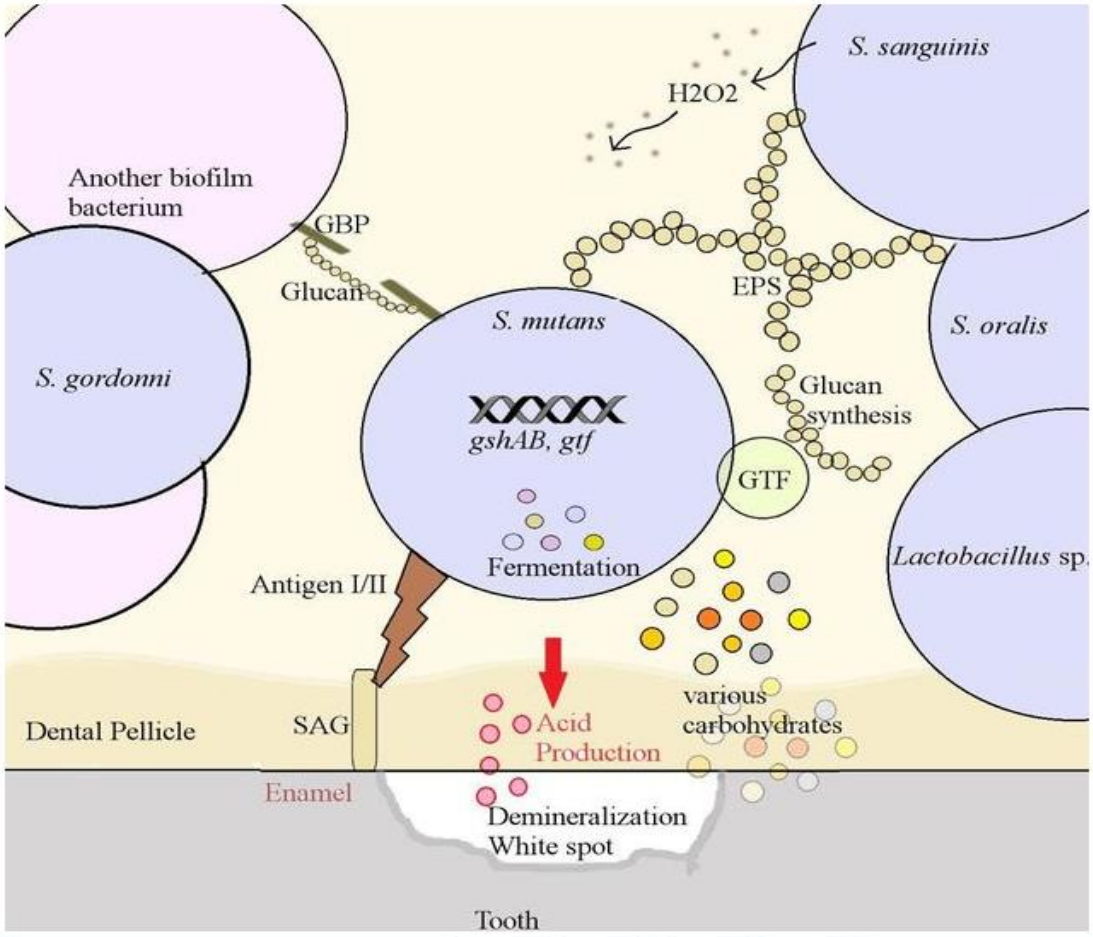


Figure (2.3): Multiple interactions in biofilm formation of *S. mutans* as described (extracellular polysaccharides (EPS), glycosyltransferases (GTF), salivary agglutinins (SAG) (Kreth J. *et al.*, 2005).

2.4.1.4 Carbohydrates Fermentation (Sugar Uptake and Metabolism)

The *S. mutans* bacterium is known for its faster fermentation of various sugars and its ability to create significant amounts of organic acids, making it more acidogenic than other oral bacteria (Marsh *et al.*, 2015). Oral bacteria absorb sugars from meals and metabolize them using glucose. They obtain the required energy through glycolysis and fermentation, resulting in the production of organic acids as byproducts (Yang *et al.*, 2016).

S. mutans can sustain their glycolytic activity in this environment, giving them a competitive edge over other oral Streptococci and enabling

them to dominate in dental plaque, leading to the development and progression of dental caries (Matsui & Cvitkovitch, 2010).

2.4.1.5 Acid Tolerance

S. mutans possesses additional virulence characteristics, including its capacity to endure alterations in its surroundings, such as low pH levels (aciduricity). The acids generated not only contribute to the demineralization of teeth, but also swiftly acidify the dental plaque milieu surrounding the bacterium. *S. mutans* is capable of adapting to pH levels as low as 4.5. The very acidic nature of this state is lethal to the majority of other microorganisms (Dinesh *et al.*, 2016).

Oral bacteria utilize the carbohydrates in the diet to generate organic acids through metabolic processes. Subsequently, the surrounding environment undergoes acidification due to the heightened concentration of protons. *S. mutans* is capable of thriving and proliferating in acidic conditions because to its many genes that allow it to withstand and adapt to such an environment. The atpD gene in *S. mutans* is responsible for encoding the F1F0-ATPase. F1F0-ATPase is a proton pump that expels H+ ions from the inside of bacteria to the external environment in order to counteract acid stress and sustain acid tolerance (Lemos and Burne, 2008).

2.4.1.6 Production of Mutacin

The synthesis of bacteriocins, known as mutacins, is a significant virulence component of *S. mutans*. The rise in bacterial resistance to antibiotics necessitates the creation of novel antibacterial compounds. Mutacins, also known as bacteriocins, are compact antibacterial peptides that are synthesized by *S. mutans* and exhibit efficacy against bacterial infections (Salh, 2014).

Bacteriocins play a crucial role in enabling certain oral bacteria to surpass others in occupying and/or preserving specific habitats. Bacteriocins are produced by ribosomes as precursor peptides including a signal peptide at the N-terminus, which is characteristic of proteins that are secreted. This signal peptide is cleaved simultaneously with the export of the fully formed peptide over the membrane. Bacteriocin synthesis by Gram-positive bacteria is typically linked to the shift from the logarithmic growth phase to the stationary phase or the cell density in the culture media (Tajbakhsh *et al.,* 2017).

Quorum Sensing QS systems are communication networks that allow microorganisms to detect and react to environmental factors including the presence of nutrients and the size of the population. This is possible because signaling molecules naturally increase as the bacterial population grows. In Gram-positive bacteria, (QS) is regulated by peptide pheromones, which operate as extracellular signaling agents that induce alterations in gene expression and ultimately lead to the activation of a synchronized response by the population (Lyon and Novick, 2004 ; Shanker and Federle, 2017). Within *S. mutans*, the presence of QS systems serves to control the production of bacteriocins, also referred to as mutacins, which are peptide antibiotics employed for defense against other oral microorganisms (Yonezawa and Kuramitsu, 2005).

2.4.1.7 Proteases

The proteases produced by *S. mutans* have a role in its pathogenicity by breaking down host proteins for bacterial nutrition and directly degrading host structural proteins (Karygianni *et al.*, 2020). *S. mutans* has demonstrated the ability to generate two extracellular proteases that can break down gelatin and substrates resembling collagen. Streptococcal

proteases may also contribute to the degradation of certain elements of the host immune system (Lindsay *et al.*, 2017).

2.5 Molecular Detection of *S. mutans*

The primary constraints of culture methods encompass a limited capacity to detect *S. mutans* in clinical samples; a variable appearance depending on the growth medium employed; and its substantial expense and labor-intensive nature. Furthermore, the process of cultivation necessitates the use of living samples, rendering it problematic for application in field epidemiology investigations and high-throughput research (Poster *et al.*, 2010).

Many various new molecular studies have been conducted to enhance the identification of target bacterial species isolates. The PCR amplification methods were extensively employed to determine the specificity of these species and are regarded as a dependable diagnostic tool for examining bacterial isolates (Al-mohammadawy *et al.*, 2018).

Many of the specialized primers targeted genes associated with virulence in *S. mutans*. Sm479 gene , is extensively utilized and has shown high success in identifying and evaluating *S. mutans* in dental caries samples (Salehizadeh, 2016). The species-specific primers and probes can be utilized for conducting large-scale epidemiological research, as well as for monitoring and evaluating the infection of *S. mutans* and its response to prevention and therapy. The specific segment being targeted includes a section of the htrA gene and a section of an intergenic region in the *S. mutans* genome. The ultimate length of the PCR amplicon is 479 base pairs.

2.6 Antibiotics Sensitivity of *S. mutans*

The increasing resistance of bacterial infections to commonly used antibiotics has become a widespread issue among humans. The proliferation of antibiotic resistance is resulting in mortality, as well as significant financial burdens. Antibiotic resistance is more widespread in low-income countries compared to developed nations (Kapi, 2014). Currently, there is a significant global interest in using antimicrobial medicines to prevent and cure dental caries, as antibiotic resistance is becoming more widespread (Mulks, 2018).

The biofilm provides a protective barrier for the bacteria against environmental stressors. The sessile cells that are present within the biofilm have a significantly higher resistance to antibiotics, up to 1000 times greater, compared to cells that are in their planktonic condition (Aqawi *et al.*, 2021).

AMP has been used widely in the clinic, and systemic administration of AMP causes resistance in oral streptococci (Fernandez and Hancock, 2012; Moffatt *et al*., 2019).

According to the Center for Disease Control of the USA, novel antibiotics are far from sufficient, and appropriate use of antibiotics could reduce the emergence of resistance (CDC, 2019).

2.7. Nano-materials

The term nano-materials mainly refers to some particles whose outer size, inner size, and surface structure are within the nano-scale (1 - 100 nm) limits, in addition to having unique physical and chemical properties and biological effects (FDA USA. 2014), which enables it to be usable in most fields, such as medical, biological, and others.

The nano-materials can reduce the concentration of the drug and its toxicity, and side effects (Ren H. *et al.*, 2018). Nano-materials synthesized by biological methods play an important role that cannot be ignored in the field of biological medicine, as in the case of diagnosis, treatment, repair, and others, compared to conventional drugs that have defects and antibiotics, to which pathogenic bacteria may develop resistance to them over time. Nanotechnology has provided good uses such as modifying and developing many important properties of metals in the form of nanoparticles (NPs) that have many applications in the field of diagnostics, biomarkers, cell labeling, antimicrobial agents, drug delivery systems and nano-drugs to treat various human diseases (Marcato, *et al.*, 2008; Singh, and Nalwa. 2011).

Nano-materials have also been used in optical devices, superconductors, biological catalysts, and biosensors (Adibkia. *et al.*, 2008; Tiwari. *et al.*, 2011). The physical and chemical properties of nanomaterials depend on their precise synthesis, synthesis methods, and the shape and size of the nanoparticle. Therefore, the effects of nanomaterials on health and the environment are also dependent on their size and shape. It is not easy to obtain one internationally accepted definition of nanomaterials, and until now the true and accurate definition of nanomaterials is under discussion in the scientific community (Patel. *et al.*, 2021).

Biosynthetic nanoparticles use biological catalysts which in turn lead to an environmentally friendly synthesis approach, thus gaining precedence over conventional physical or chemical methods (Kruis. *et al.*, 2000; Hebbalalu. *et al.*, 2013).

There are many applications for nanomaterials, such as transporting and loading drugs effectively due to their large specific surface area, and the possibility of using them as antibiotics that kill pathogenic bacteria, and nano-materials have good biocompatibility and have biodegradability, and they can accumulate in human organs with fewer side effects (Qiu M. *et al.,* 2019).

2.8 Biosynthesis of Nanoparticles

The biosynthesis of nanoparticles by various biological methods is considered a major attraction factor through which chemical toxicity and harmful side effects that generally result in chemical methods can be avoided (Karthik *et al.,* 2013).

Recently, many environmentally friendly factories have been established, where the synthesis of nanomaterials (by various biological methods) has been recognized as a potential means of nanosynthesis. The biosynthetic processes of nanoparticles within the desired ideal properties that are validated make them widely applicable in various fields such as solar energy absorbers, interfacial materials in electric batteries, labeling and biological sensors, catalysis in chemical reactions as well as their utilization in many biomedical agents and treatments (Klaus-Jeorger *et al.*, 2001; Duran. *et al.,* 2005).

The biological sources adopted in the synthesis of nanoparticles are multiple according to the type of organisms that are used as raw materials in the synthesis process, whether they are plant, bacterial, fungal, viral, parasitic, or any vital part through which nanoparticles with standard characteristics can be obtained. It provides a different, environmentally friendly, highly efficient and effective treatment method. The process of nanoparticle synthesis by biological methods is called green synthesis of

nanoparticles. They have a well-defined and controllable size, they are free of pollutants, they are considered facile and they are widely used in medical and biological applications, these are some of the additional benefits of green synthesis, figure (2.4) (Mittal *et al.*, 2013).

It is possible to determine the biological activity of biologically manufactured nanoparticles to a large extent and control them by the green material, which is the raw material used to stabilize and reduce metal ions, as well as the importance of careful care of the steps involved in the synthesis process. An ideal characteristic of nanoparticles should be that they should have a distinct ability to discriminate potential targets (pathogens) and mammalian (host) cells (Rónavári *et al.*, 2013).

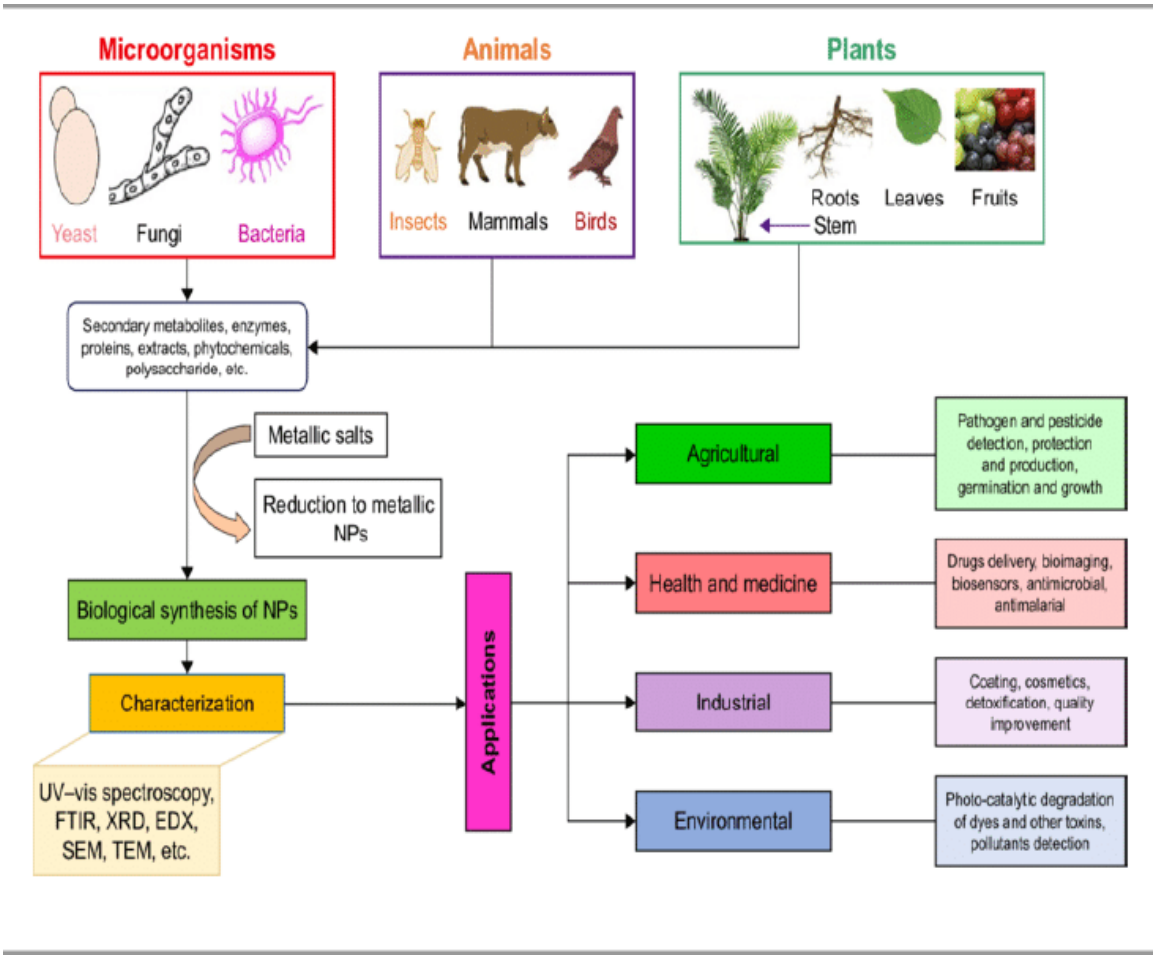


Figure 2.4: Schematic diagram for showing green biosynthesis nanoparticles from different biological sources and there applications (Rónavári *et al.*, 2013).

2.9. Characterization of Nanoparticles

2.9.1 Scanning Electron Microscope (SEM)

Scanning electron microscopy (SEM) can be employed for the assessment of elementary structure, including grain size, surface roughness, porosity, dimension distribution, homogeneity, inter-metal distribution, and diffusion of nanoparticles (Van Malderen, 2017).

The underlying principle of the mechanism of action involves the utilization of a finely focused scanning electron beam that traverses the surface of the sample. This process results in the production of backscattered electrons, secondary electrons, and distinctive X-rays. According to (Goldstein *et al.* 2017), the signals obtained by detectors are utilized to generate images of the scanned sample, which are then visualized on a cathode ray tube screen.

2.9.2 X-Ray Diffraction (XRD)

The methodology of (XRD) analysis is commonly referred to as a radiation diffraction method (Spiliopoulou *et al.*, 2020). There exist numerous techniques for the examination of three-dimensional structures, among which x-ray crystallography has been widely regarded as the most efficient method. In addition to amorphous substances, such as polymers, X-ray diffraction is employed as a technique for elucidating the atomic configurations and measuring the thickness of thin films (Maveyraud and Mourey, 2020).

The utilization of the microscope entails a two-step process in the initial stage, incident light interacts with the target and undergoes diffraction, resulting in its dispersion in several directions. Subsequently, the lens intercepts and reconstructs the diffracted rays, so generating an

image. X-ray technology was employed for the purpose of detecting molecular diffraction, with the aid of a screen to reconstruct the resulting image (Oake. *et al.,* 2019; In-Hang , 2012.).

2.9.3. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectroscopy is a technique based on vibrational spectroscopy that yields a clear image to study the properties of a variety of chemical compounds when the sample to be detected is a powder when shed in the middle infrared (mid-infrared), vibrations are generated Many important molecules, as in organic compounds, metal phases, and oxyanions. FTIR spectroscopy can be used as a quantitative analytical method for solids and compounds to determine the mechanisms of bonding between them, these characteristics are identified by using FTIR spectroscopy (S\700, Nicolet, MA. USA) (Lesiak *et al.,* 2019). The molecular vibrations of organic compounds lead to the formation of distinct absorption bands in the spectra, and this helps to detect them (Hospodarova *et al.,* 2018).

2.10 Iron Nanoparticles (Fe NPs)

Iron is one of the metals that is widely used because of its crystal structures, low cost, magnetic properties, different oxidation states, and environmental friendliness in nature. Iron nanoparticles are used in many medical and biological applications, as they are considered safe agents and do not negatively affect the epithelial lining of cells in the field of biomedicine, as well as their magnetic ability to deliver the drug to the target place or organ, and being an antimicrobial and antioxidant agent (Arakha *et al.,* 2015).

There is a bonding relationship between iron nanoparticles and oyster shell, as it gives a support to it by the wet impregnation technique (Petala *et al.,* 2013). A researchers collected oyster shells to research

laboratories, and after cleaning them by removing mud from them, rinsing them for 10 minutes, and washing them with deionized water, They crushed and dried them in ovens at a temperature of 100 C , and then sifted them with a mesh of 100 mm. The iron nanoparticles were prepared using the dissolved iron reduction method, which consists of four stages; Mixing, separation, washing and drying (Pan BC. *et al.*, 2005, Wan Ngah WS. *et al.*, 2004).

2.10.1 Antibacterial activity of Fe NPs

Iron is one of the metallic elements that can be found in the greatest abundance on Earth. Recently, through recent research studies they have been recognized as a new class of mainstream nanoparticles due to their unique properties, including high covalence, super para-magnetism, catalysis, sensors, and antibacterial activity, drug delivery, biomedicine, as well as the use of iron nanoparticles (Fe NPs) in environmental cleaning applications (Miguel *et al.*, 2020).

The most important characteristic of nanoparticles is that they perform superior activities in wide-ranging fields especially the biosynthesis of nanoparticles (Rónavári *et al.*, 2021). For the synthesis of different types of nanoparticles, there are a variety of physical, chemical, biological and hybrid methods (Naseem *et al.*, 2015). Small sized iron nanoparticles have a larger surface area which causes conformational changes in the DNA of the bacterial cell, so it acts as an antibacterial. Gram +ve bacteria have a thick peptidoglycan membrane, which is extremely difficult for iron nanoparticles Fe NPs to penetrate (Nwamezie *et al.*, 2018). Another mechanism of iron nanoparticles Fe NPs and their antibacterial activity is through the generation of reactive oxygen species (ROS), as shown in figure (2.5), a phenomenon that occurs due to the

Fenton reaction of iron and its metabolic products (Shahnaz Majeed. *et al.*, 2021; Gabrielyan, L. 2019).

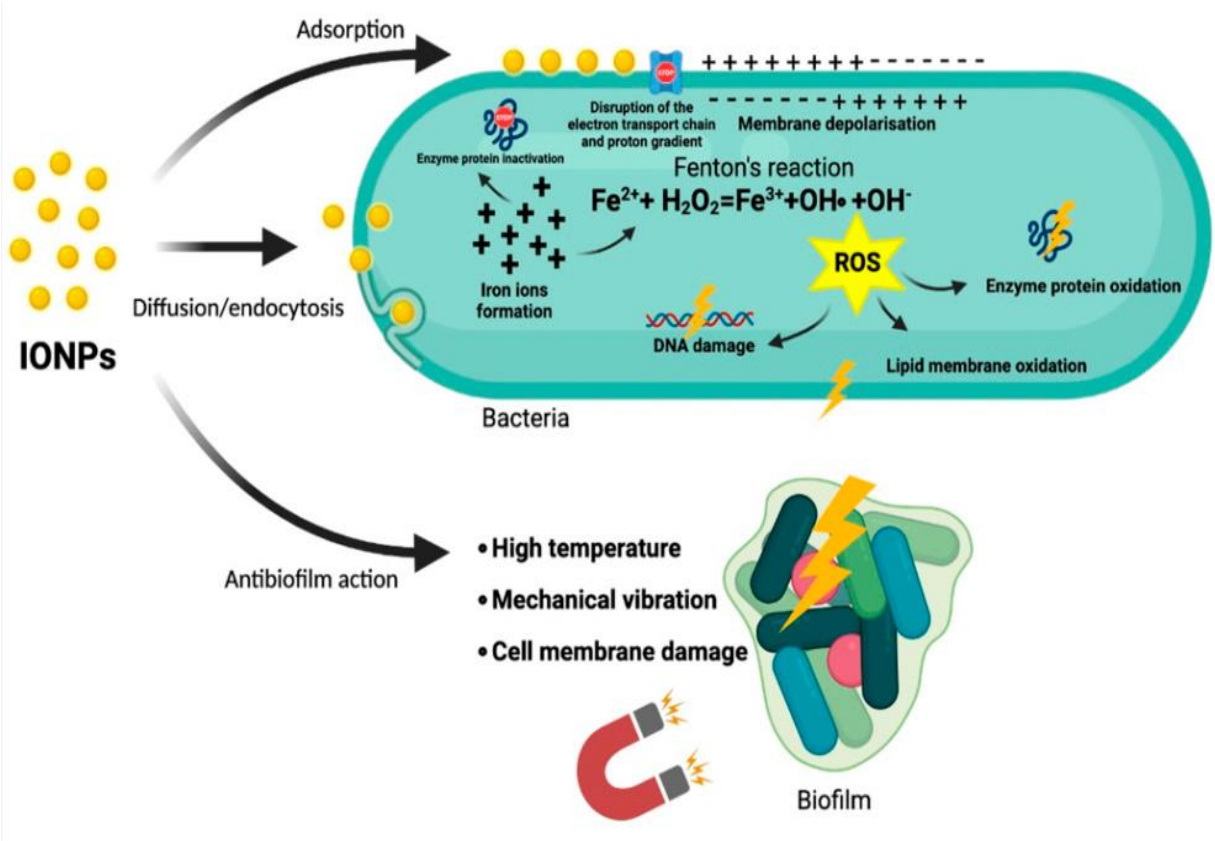


Figure (2.5): The mechanisms of IONP antibacterial activity of FeNPs (Sergey V. Gudkov, *al.*,. 2021).

In the study conducted by (Xu Weihua. *et al.*, 2022) it was demonstrated that the application of iron nanoparticles in environmental remediation has significant effect on the environmental pH value, the pollutant morphology and the toxicity to microorganisms.

According to the study of (Vitta Yosmery, *et al.*, 2020) obtained iron nanoparticles (FeNP) from the aqueous extract of the leaves of Eucalyptus robusta and to evaluate their antimicrobial activity on different pathogenic microorganisms, Subsequently, the antimicrobial activity was evaluated against the microorganisms *Pseudomonas aeruginosa*, *Escherichia coli*، *Staphylococcus aureus* and *Bacillus*

subtilis, being a close relationship between the conditions of synthesis and the increase or decrease of the antimicrobial activity.

In the study conducted by (Al-Maliki Q. A. and Taj-Aldeen W. R. 2021) supernatant of gram positive *Bacillus Coagulans* was employed in synthesis of IONPs as stabilizing and bio reducing agent, in avenge about 15.2 nm , and to reveal the activity of IONPs as an antibacterial and antibiofilm formation agent respectively against Uropathogenic *E.coli* bacteria by well diffusion method in depends on concentration.

2.11 Gold Nanoparticles (Au NPs)

Nano materials are becoming more widely used in medicine due to their drug delivery mechanism in cancer therapy, as well as their availability, material properties, and capacity to improve drug selectivity against cancer cells (Fu LH. *et al.,* 2017; Liu F. *et al.,* 2019). Gold nanoparticles (Au NPs) have generated increased interest among diverse metallic nanoparticles because of their unique qualities, which include nano-size ,low toxicity, comparatively simple fabrication, and precise targeting (R. Groning. *et al.,* 2001). The antibacterial property of Au NPs has recently been a major research topic, making them a good candidate for antibiotic complementation.

The study conducted by (Abdulazeem, L. and Abd, F.G. 2019) involve the reduction of gold nanoparticles (AuNPs) from biological sources of bacteria, fungi, and plants, as it attributed as eco-friendly and contributed to the application in nanotechnology, using the culture supernatant of local *Serratia spp*. isolate. Gold(III) chloride trihydrate (HAuCl2) in concentration1× 10^{-3} M added to supernatant separately.

Their respective supernatants were examined for the ability to produce gold nanoparticles.

In other study related to (Abdulazeem, L. and Abd, F.G. 2019) the green biosynthesis for nanoparticles was carried by using local strain *Escherichia coli* by adding Hydrogen tetra chloroaurate to growth of bacteria in brian heart infusion. The results found the ability of bacteria to synthesis AuNPs by change the color to purple as qualitative test and confirmed byusing X-Ray diffraction the peak was in 38 that detect gold nanoparticles, the Field Emission-Scanning Electron microscopes (FE-SEM) measurements appeared diameter 29.71 nm, Fourier Transform Infrared (FTIR) the results found the ability of bacteria to synthesis AuNPs.

2.11.1 Antibacterial activity of (Au NPs)

The antibacterial activity of Au NPs is mediated by the development of holes in the bacterial cell wall, resulting in cell death due to the loss of cell contents. Due to high biocompatibility, chemical stability, convenient surface bio-conjugation with molecular probes, excellent surface plasmon resonance and low toxicity, biosynthesized gold nanoparticles have diverse biomedical applications including drug delivery, cancer treatment, DNA-RNA analysis, gene therapy, sensing and imaging, antibacterial agent etc. (Paciotti. *et al.*, 2004; Lee *et al.*, 2020).

Au NPs are becoming promising cancer therapeutic and diagnostic metal NPs that attract researchers due to their unique physiochemical properties such as stability, biocompatibility, high thermal activity, optical, electrical, high surface area to volume ratio surface chemistry, and multi functionalization, etc., by fine tuning the components and concentrations, Au NPs can be easily manufactured into various forms

and sizes (Arshad, R. *et al.,* 2021; Koushki, K. *et al.,* 2021). Au NPs have also shown significant advancement in treating inflammatory diseases and bacterial infections (Piktel, *et al.,* 2021; Cui, *et al.,* 2012).

Au NPs can interact with bacterial cell membranes, destroying their integrity and playing an antibacterial and sterilizing role (G. Chu and Y. 2018). Studies reported that AuNPs protected by 2nm core cationic monolayers can interact with the cell membranes of Gram-positive and Gram-negative bacteria, forming unique aggregation patterns and bacterial cell lysis, indicating that the cationic surface properties of 2nm AuNPs can be used as antibacterial agents (S. C. Hayden, *et al.,* 2012), as shown in figure (2.6).

Antibacterial activity of AuNPs was investigated using the standard well diffusion method against *Bacillus subtilis*, *E. coli*, and *K. pneumoniae* (Sathiyaraj *et al.,* 2020). Freshly formed culture was spread on sterilized nutrient agar plates, and 8mm wells in the agar plates were made with a sterile cork borer. The wells were loaded with different concentrations (10 µL, 20 µL, 30 µL, and 40 µL) of AuNPs and 10 µL of ciprofloxacin was serving as a control. After that, the plates were maintained at 37◦C for a day, and the antibacterial activity of the AuNPs was determined by measuring zones of inhibition (ZOI) around the well impregnated with AuNPs.

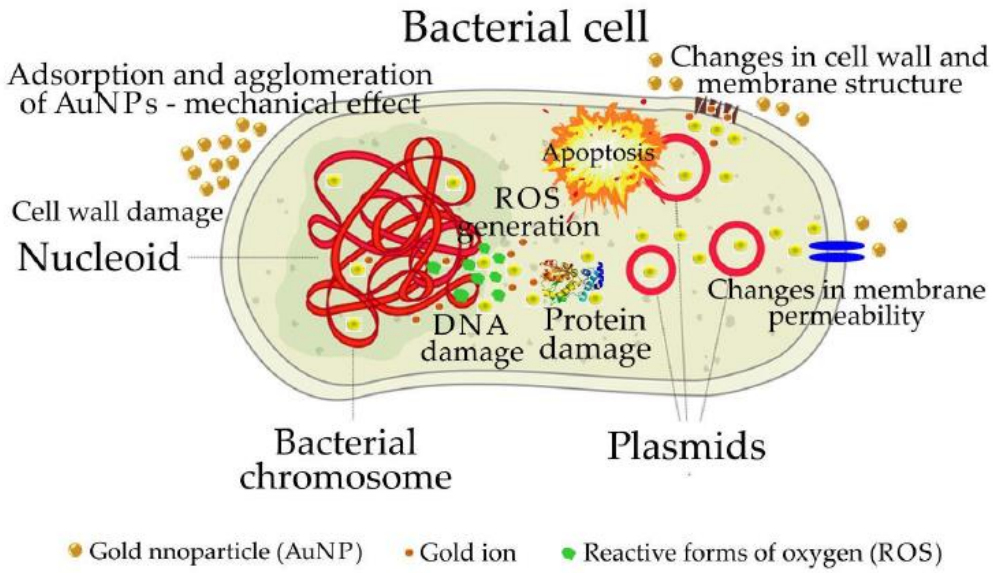


Figure (2.6): Mechanism of antibacterial and antioxidant action of biosynthesized Gold nanoparticles (AuNPs) (Nie, Z. *et al.*, 2007).

2.12 Silver Nanoparticles (Ag NPs)

Silver was known only as a metal until the recent advent of the nanotechnology era, when it became recognized that silver could be produced at the nano-scale (Silver S. *et al.*, 2006). Ag NPs are the basic essential elements in the wall of nanotechnology and it exhibits fabulous advanced characteristic features based on their properties such as size, morphology and other size dependent properties (Fayaz *et al.*, 2010).

Moreover, these nanoparticles have drawn the attention of researchers because of their extensive applications in areas such as mechanics, optics, biomedical sciences, chemical industry, electronics, space industries, drug gene delivery, energy science and catalysis (Liu, *et al.*, 2013).

In the study conducted by (Baqer J. M. and Taj-Aldeen W. R. 2021) the biosynthesize of silver nanoparticles performed by *lactobacillus acidophilus* isolates were cultured on De man rogosa sharpe broth and agar for 24 h at 37°C.Based on the biochemical tests and the fermentation

test for carbohydrates distinguished the *Lactobacillus acidophils*, the method is gaining an extremely significant field due to its economic and environmental advantages compared to the chemical and physical methods of synthesis, by adding silver nitrate (AgNO3) to the cell-free supernatant per *Lactobacillus acidophilus* at a concentration (5 mM) to be used as a precursor for the synthesis of Ag NPs.

2.12.1 Antibacterial activity of (Ag NPs)

Some studies indicate that the antibacterial mechanism of the silver nanoparticles (Ag NPs) relays on their ability to penetrate the bacterial cell wall, causing direct and indirect lipidic peroxidation, which damages the cell membrane, disrupts the DNA replication, and repairs and inhibits the respiratory protein, as shown in figure (2.7) (Martinez-Gutierrez *et al.,* 2012). The use of silver nanoparticles both as antimicrobial agent (Majeed *et al.,* 2016) and as a potential drug carrier in treatment of cancer has recently gained considerable attention (Nayak *et al.,* 2016).

In the study conducted by (Hamza F. K. *et al.,* 2021) antibacterial activity of biosynthesized AgNPs against multidrug-resistant bacteria (MDR) of both gram-positive and gram-negative bacteria, as well as biofilm formation of *Klebsiella pneumonia* isolates were collected from clinical samples of urinary tract infection.

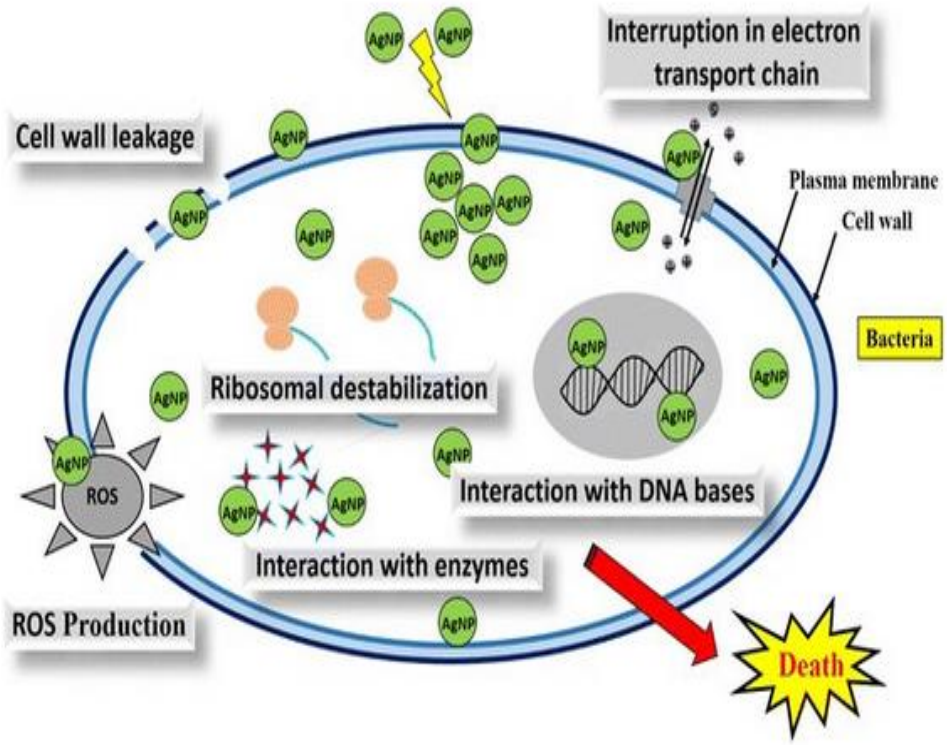


Figure 2.7: Mechanisms of antibacterial activity for Ag NPs are diagrammatically representation (Kharat and Mendhulkar, 2016).

Chapter Three
Materials and Methods

3. Materials and Methods.

3.1. Materials.

3.1.1. Equipment and Instruments

The equipment's and instruments used in this study, as well as their manufacture companies and origin, are listed in the table (3-1).

Table (3-1): Equipment's used in present study.

Equipment and Instruments	Manufacture company	Origin
X ray diffraction (XRD)	Broker	Germany
Autoclave	Labtech	Korea
Balance (electrical)	Denver	Canada
Centrifuge	Hitachi	Japan
Digital camera	Sony	Japan
Distillator	GFL	Germany
Fourier Transform Infrared (FT-IR)	Perkin-Elmer 1725x	Japan
Hood	Bio Lab	Korea
Incubator , Oven	Memmert	Germany
Light Microscope	Olympus	Japan
Micro and cooling centrifuge	Hermle Labortechnik	Germany
ELISA system	Beekman	Austria
Micropipette	Eppendrof	Germany
pH meter	Orient	USA
Refrigerator	Beko	Korea
Scanning electron microscope	FEI	Netherland
Shaking incubater	Gallenkamp	England
Thermocycler	Bio- Rad	USA
UV-visible spectrophotometer	Shimadzu	Japan
Vortex mixer	Thermolyne	USA

Magnetic Stirrer	Digital M.S	USA

3.1.2 Chemical and biological materials

The Chemical and biological materials used in present study are listed in (table 3.2)

Table (3.2): Chemical and biological materials used in this study.

Materials	**Manufacture company**	**Origin**
Agarose	Conda	USA
Crystal violet powder	BDH	England
DNA Ladder 100 bp	Promega	USA
DNA loading dye	Promega	USA
Ethanol (96%)	BDH	England
Ethidium Bromide Solution	Bio Basic	Canada
Glycerol	Sigma	USA
Gram stain	Himedia	India
Gold Chloride ($AuCl_3$)	Photographers' Formulary	USA
Hydrochloric acid (HCl)	EMC	Germany
Methanol	BDH	England
Phosphate buffer saline (PBS)	Bioworld	USA
Silver Nitrate (AgNO3)	Forbes Pharmaceutical	India
Sodium hydroxide (NaOH)	EMC	Germany
Sucrose	Himedia	India
Tris-EDTA (TE) buffer	Bio Basic	Canada

3.1.3 Culture Media

The culture media which used in the present study and the purpose from used it listed in table (3.3).

Table (3.3) : Culture media that used in this study with their purpose.

Culture Media	Company/origin	Purpose
Nutrient agar	Himedia /India	used for the growth and isolate bacterial types
Mitis – Salivarius agar	HIMEDIA	India
Brain heart infusion broth BHIB	Himedia /India	Used for preservation of bacteria
Brain heart infusion agar BHIA	Himedia /India	Used for growth ,activation and maintenance of bacteria
Muller Hinton Agar	Lab /USA	Determine the sensitivity test of bacteria to antibiotic

3.1.4. Antibiotic discs

The antibiotics used in this study are listed in table (3.4).

Table (3.4): Antibiotic Disc , Symbol and Potency.

Antibiotic Group	Antibiotics	Symbol	Concentration (µg\disc)	Company \ Origin
Cephalosporins	Cefixime	CFM	5 mcg	Liofilchem\Italy
	Ceftazidime	CAZ	10	Bioanalyse\Turkey
	Ceftriaxone	CRO	10	Bioanalyse\Turkey
	Cephalosporin	CEP	10	Liofilchem\Italy
Macrolides	Erythromycin	E	10	Liofilchem\Italy

Penicillins	Piperacillin/tazobactam Rifampicin	PZT RA	110 5	Liofilchem\Italy
Monobactam	Azithromycin	AZM-	15	Bioanalyse\Turkey
Tetracyclins	Doxycycline Tetracyclin	DA TE	10 10	Liofilchem\Italy Liofilchem\Italy
Fluoroquinolones	Levofloxacin Ciprofloxacin Fluorinated ethylene propylene	LEV CIP FEP	15 10 10	Bioanalyse\Turkey Bioanalyse\Turkey Bioanalyse\Turkey
Bactrim	sulfamethoxazole	SXT	25	Pharmaceutical Press\UK
Carbapenenems	Meropenem Imipenem	MEM IPM	10 10	Bioanalyse\Turkey Bioanalyse\Turkey
Quinolone	Nalidixic acid	NA	30	Niksan Pharmaceutical\India

3.1.5. Primers

PCR primers for quantification gene expression (Sequence of Sm479 gene, forward and reverse, and master mix) detection in *Streptococcus mutans* were used according to the study of (Chen Z, *et al.*, 2007).

3.2 Methods

3.2.1 Study Design

Steps of the research project were shown in figure (3.1).

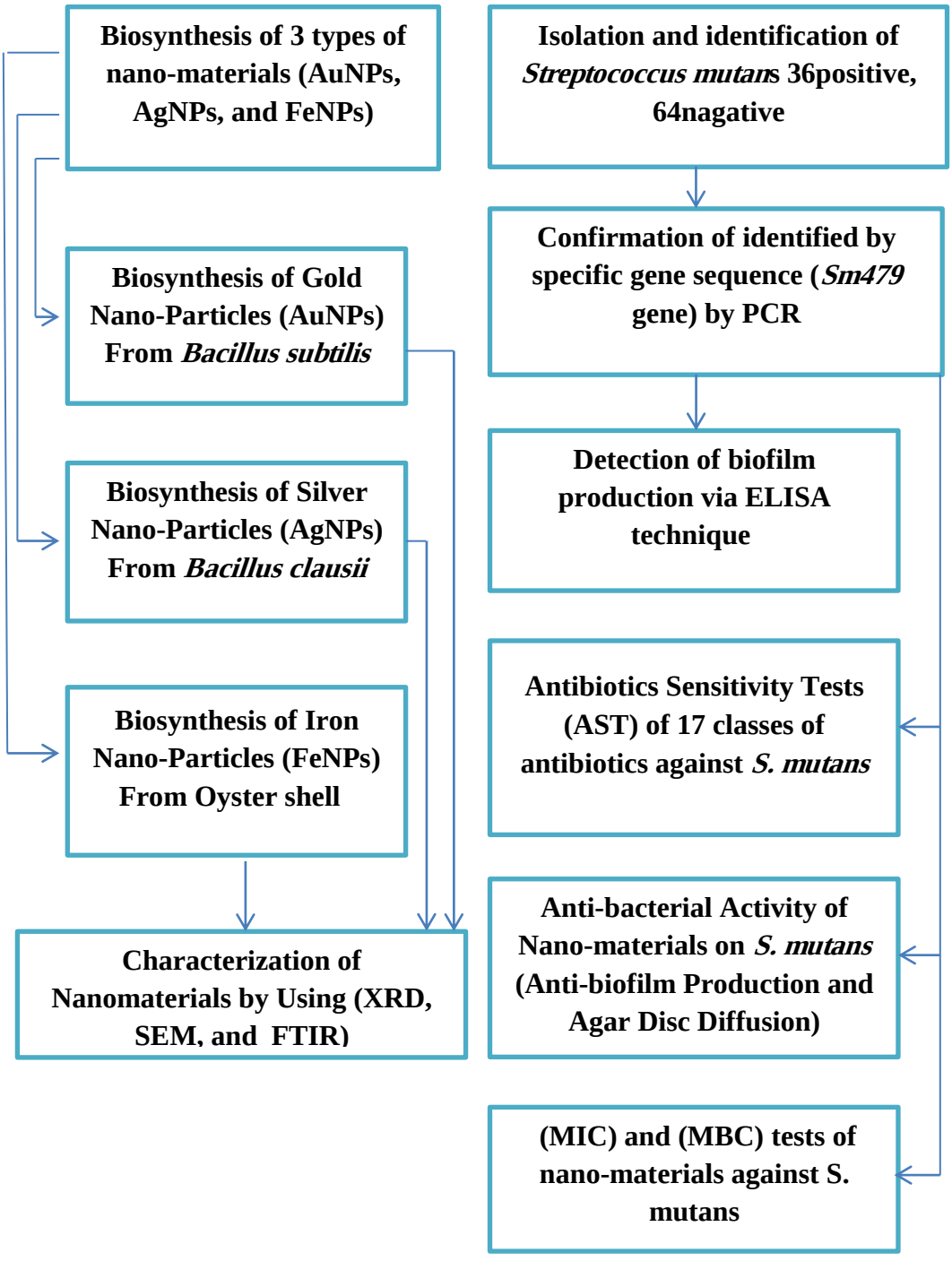


Figure (3-1): Schematic diagram representing the study project.

3.2.2. Collection of sample

Hundred samples of dental caries were collected by swab transport media (invasive sterile collection), from patients that they visit the Educational Clinics of the College of Dentistry / University of Babylon, during (April \ 2022 to August \ 2022). And they cultured by using (Mitis Salivarius Agar Base) with the addition of (2 U/ml of Bacitracin, and 2 gm of 1% potassium tolerate).

3.2.3 Media preparation and sterilization technique

All culture media were prepared according to the manufacturing company instructions .The constituents were dissolved in distilled water completely , sterilized by autoclaving at 121Cº for 15min at 15 pound/inch2. After cooling to 45-50 C° each medium was dispensed into sterile petri dishes in case of agar media and in sterilized screw tubes in the case of broth media. The work performed in laminar flow cabinets. Then , poured media were incubated for 24hr at 37 C° to ensure sterility (MacFaddin J. F. 2000).

3.2.4. Preparation of Reagents & Solution

3.2.4.1. Phosphate Buffer Saline (PBS)

Phosphate buffer saline was prepared by dissolving 1 tablet of (PBS), PH 7.2 in 100 ml deionized distilled water (DDW). It was used in biofilm and anti-biofilm experiments (Collee, J. *et al.*, 1996).

3.2.4.2. Hydrochloric acid (0.25 M)

It was prepared by adding 20.444 ml of 37.5% hydrochloric acid , and slowly dilute to 1 liters D.W. in a volumetric flask. Then allowed the

solution to cool to ambient temperature and properly mix it (Chen, M. Collee, J. *et al.*, 2020).

3.2.4.3. Sodium Hydroxide (NaOH) Solution (1M)

Sodium hydroxide (1M) was prepared by dissolving 4g of NaOH up to 50 ml of distilled water; this solution was used to adjust the pH during pH optimization step (Baker, and Silverton, 2014).

3.2.4.4. TBE Solution

TBE buffer was prepared as 10x by Promega company. Tris-borate-EDTA buffer used at concentration of 1 X (1: 10 dilution of the concentration stock). The stock solution was diluted by D.W. and stored at room temperature.

3.2.4.5 Crystal Violet Solution(1%)

Crystal violet solution was prepared by dissolving 1gm of crystal violet in 100 ml Distilled water (Vandepitte *et al*., 2003).

3.2.5. Preservation and maintenance of bacterial isolate

The preservation and maintenance of isolates were done according to (Silhavy, T. *et al.*, 2010) as follows:

3.2.5.1. Short term preservation

The positive cultures were stored on nutrient agar at 4C° until further testing.

3.2.5.2. Long term preservation

Fresh 24 hr nutrient agar cultured isolates frozen in 20% glycerol brain –heart infusion broth and stored at -20 C° until required.

3.2.6. Morphological Identification of Isolates

The specimens were spread on the modified Mitis Salivarius Agar Base with the addition of 2 U / ml of Bacitracin and 2gm of 1% potassium tolerate, and purified on the same agar media by sub-culturing of isolates (MacFaddin, 2000). then samples were taken from them by swab, and placed on a slide with distilled water (or injection water), in order to prepare them for the morphological examination using light microscopy after performing Gram Stain method, as shown in figure (3.1).

3.2.7. Molecular Identification of *Streptococcus mutans*

Amplification specific gene sequence (Sm479 gene sequence) test by PCR technique has been used in this study for the identification of *Streptococcus mutans* (Chen Z, *et al.,* 2007).

3.2.7.1. Extraction of Bacterial Genomic DNA

Total DNA was extracted from culture broth, 1.5 ml of culture broth pipetted into Eppendorf tubes then centrifuged at 1.500 rpm for 5 min and the supernatant discarded , 200 µl of TE buffer was added , vortexes well , boiled for 15 min in water-bath , and then put on ice immediately for 1 min , this is centrifuged again at 1.500 rpm for 10 min and the supernatant was collected, which contains DNA for use as DNA template (Suwanjinda, D. *et al.*, 2007). DNA concentration and purity was estimated by nanodrop at absorbance (260 /280 nm) , DNA integrity was detected in Agarose gel electrophoresis (Bunu, S. J. *et al.*, 2020).

3.2.7.2. The mixture of Polymerase Chain Reaction PCR reaction

Polymerase chain reaction has been used to amplification Sm479 gene sequence as show in table:2 according to Promega manufacturing instruction, by adding material in table:3 to PCR tube and the final volume 20 μ l. PCR green master Mix (Promega/ USA) has been divided into aliquots into individual PCR tubes (each aliquot was 20 μ l) and all the reaction components were kept on ice. 2 μ l of forward and reveres primers were added. 3 μ l of DNA pattern was added to the PCR Super Mix. All These components has renounced in ultracentrifuge at 16000xg for 2 minutes. The reactions were placed in a thermal cycler that had been preheated to 95 C° , and previously set up to the following cyclic conditions table:3.3.

Table 3.5: Sequence of Sm479 gene (Chen Z, *et al.*, 2007):

Sm479	Sense	5 ‘ TC GCGAAAAAGATAAACAAACA 3 ‘
	antisense	5 ‘ GCCCCTTCACAGTTGGTTAG 3 ‘

Table 3.6: Show content of PCR tube:

Chemical compound	Volume
Master mix	5 µ l
DNA	3 µ l
Sense primer	1 µ l
Antisense primer	1 µ l
Deionizer D.W	10 µl
Total	20 µl

Table 3.7: PCR condition for sm479 gene:

Step	Temperature C°	Time mine	Cycles
Initial	94	5	1
Denaturation	94	4	30
Annealing	56	1	30
Elongation	72	2	30
Final Elongation	72	7	1

3.2.7.3 Detection of Amplified Products by Agarose Gel Electrophoresis

Gel electrophoresis was used for detection of DNA , 1.5 gm of Agarose was weighted and applied to 100 ml of TBE (1X) buffer and microwaved until the solution became clear. After cooling the agarose to 50 C°, 5 microliter of ethidium bromide dye was added to 100 ml of melted agarose gel , and poured on preparing tray. Comb was removed after the hardening of agarose leaving wells , 5-10 microliter of DNA was mixed with 1-2 microliter of loading dye , TBE (1X) buffer was added to the electrophoresis tank. A tray with agarose was immersed in electrophoresis tank. Each well was loaded with 6 microliter of DNA sample and standard molecular weight of DNA ladder (100-1500 bp) were loaded in a first well. Electrophoreses ran at 75 volt for 40 min . Gel was visualized with UV transilluminator at 280 nm and photographed by using digital camera (Mishra, P. *et al.*, 2009).

3.2.8. Detection of Biofilm Formation

The estimation of biofilm formation by *S. mutans* isolates was conducted using the microtiter plate crystal violet method, as described in studies of (S. Stepanović *et al.*, 2000; Y. Zhou *et al.*, 2018).

- The isolates of *S. mutans* from the glycerol stock were cultured on brain heart agar media and incubated at 37ºC in a candle jar for 48 h. A sterile plastic loop transferred a loopful of bacterial colonies into a can-tube containing 5 ml of isotonic saline and adjusted to match the 0.52-0.62 McFarland turbidity standard.
- Then, 100 µl from the standardized isotonic saline was transferred into 10 ml brain heart broth and 200 µl of each of the diluted solutions was transferred to a sterile flat-bottom 96-well plate containing 100 µl of fresh media per well (Brain heart infusion broth + 5% sucrose) in triplicates and incubated at 37ºC in a candle jar for 24 h. The negative control wells contained all components except the bacteria.
- Following incubation, the broth was removed, and the wells were gently washed three times with saline solution. 200 µl Phosphate Buffer Solution (PBS) was added to each well for exception of control (this step is repeated twice and removed immediately), subsequently 200 µl sodium acetate was added to each well with control for half hours (fixation of bacteria).
- After removing of sodium acetate from each well without washing, the plates were left to dry, then followed by biofilm quantified using 200 µl of 0.1% crystal violet for 15 min.
- The excess stain was washed off by saline solution and inverted on tissues and left to dry, after that resolubilized by 100 µl of 98% ethanol for 15 min. The optical density (OD) was measured at 570

nm by Stat Fax-2600 microplate reader (Awareness Technology, USA).

The results recorded as shown in Appendix (1) by the microtiter plate followed according to (S. Stepanović *et al.* 2000). The cut-off optical density (ODc) for the negative control was established as follows:

1. OD $\leq$ ODc indicate non-adherence.
2. ODc < OD $\leq$ 2 ODc indicate weak adherence.
3. 2ODc < OD $\leq$ 4 ODc indicate moderate adherence.
4. OD > 4 ODc indicate strong adherence.

3.2.8. Antibiotic Sensitivity Test (AST)

Streptococcus mutans previous cultured colonies were activated by plated it on brain heart infusion (BHI) agar , the cultural characteristics of *S. mutans* were noticed after an incubation at 35 - 37°C for 48 hours as white to grey coloured colonies of an average size of 1 mm in diameter (Wormser and Stratton, 2007). Inoculum was prepared from each culture based on established protocols (Hudzicki, 2009).

Kirby - Bauer method was accomplished to complete this test according to the Jubair (2015). Isolates have streaked on Muller ‑ Hinton agar and the antibiotic discs were placed equally spaced intervals on the surface of the medium with flamed forceps or a disc applicator and left for 15 minute, incubation was usually overnight with an optimal time of 14 hours at 37C$^{\circ}$.

3.2.9. Biosynthesis of iron nanoparticles (Fe NPs)

The iron was prepared without adding iron salt, as it is already present in the composition of the oyster shell. The construction of the oyster shell

supported by iron nano-adsorbent, that possible to obtained by wet impregnation technique (Petala E. *et al.*, 2013; Y Wan, *et al.*, 2012).

The biosynthesis of iron nanoparticles (FeNPs) method include collecting, cleaning, disinfecting, breaking down, grinding of oyster shell for transformed it to powder (Pan BC. *et al.*, 2005; Wan Ngah WS. *et al.*, 2004), and then followed the following steps:

1. Fifty ml of deionized water is added to 10gm of oyster shell powder, and then kept for one day at room temperature.
2. 10% HCL were prepared by adding 100ml deionized water to 10ml HCL.
3. Hundred ml of 10% HCl was added to the preserved mixture that contains 10gm oyster shell powder + 50ml deionized water, and all mixture was kept or 4 hours.
4. The mixture that resulted from the previous step was filtered by filter paper after four hours of mixing in the magnetic stirrer rotor device.
5. Ten of 10gm sodium hydroxide was added to 100ml of deionized water and covered for one day, then the remaining powder that resulted from the previous step was added.
6. The mixture was shaken by the magnetic stirrer device for about 4 hours, and the iron particles were attracted to the metal capsule that rotates inside the beaker that was placed on the magnetic stirrer device.

7. Iron nanoparticles FeNPs that attracted around capsule were collected for preparing and examine them by XRD, SEM, and FTIR examination.

3.2.10. Biosynthesis of gold nanoparticles (Au NPs)

1. *Bacillus subtilis* prepared in advance in tube of (Brain Heart Infusion Broth). (100 microliters) of (Brain Heart Infusion Broth) that contained bacteria (stored in the refrigerator as stock) was taken and added to (10 ml) of broth , and incubated for (24 hours) at (37 C). This step is just an activation of the bacteria, which become 24 hours age.
2. One ml of the activated bacteria was added to 1000 ml of the broth, and then it was incubated at 37 C, for a period of 48 to 72 hours, in order for the primary and secondary metabolites to be sorted by the bacteria, because they are the ones that give the nanoparticles.
3. Sixteen ml of 5% AuCl was added into 40ml of the bacteria that prepared by advanced step, then two drops of sodium hydroxide NaOH were added to the mixture, to ensure that there is no aggregation of gold nanoparticles to be prepared.
4. Whole component, after being covered by silicon cover (to avoid oxidation by light, is placed on a magnetic stirrer device 100 rpm, for 24 hours, at 20 C, then examinations done.

3.2.11. Biosynthesis of silver nanoparticles (Ag NPs)

1. Thirteen gm brain heart agar added to 1000ml of DW with shaking to become clear, then sterilization by autoclave.
2. Cultivation of *Bacillus clausii* isolates as stock on Brain Heart Broth, followed by incubated at 37°C for 48-72 hours, to give secondary metabolites.

3. The broth were centrifuged at 500 rpm, for 20 minutes.
4. The supernatant is taken, it represented the secondary metabolites, which are the ones that interact with the compound used for the production of silver nanoparticles Ag NPs and the precipitate must be ignored.
5. Ten mg AgNO3 was added to 600ml of the resulting supernatant, then placed on a magnetic stirrer device at about 300 rpm, for 30 minutes (Stir Bars., 2014). This device is used to make a stir bar, immerse in a liquid, quickly spin, or stirring and mixing a solution.
6. Whole amount were placed in a shaker device at room temperature 37C from one to two days, the color change is monitored, and noting the PH it must be alkaline (PH=9), (measured by filter paper).

3.2.12. Characterization Of Nanoparticles

The physical characteristics of (FeNPs), (Ag-NPs), and (Au NPs) were characterized by XRD, SEM, and FTIR.

3.2.12.1 X-Ray Diffraction (XRD) analysis

The working principle of the XRD method involves the scattering of X-rays due to the revolution of electrons in the atom's nucleus when the rays strike on the nanoparticles. The scattered X-rays are reflected in various directions, which cause interference patterns. These patterns are either destructive or constructive (He S. *et al.*, 2007) but only the scattered X-rays that undergo constructive interaction result in diffraction. The XRD patterns were collected on Bruker AXS D8 Advanced X-ray diffractometer with Cu Kα radiation and scanning angle 2θ over the range of 10-80°. XRD patterns were calculated using X'per Rota flex diffraction meter using Cu K radiation and λ=1.5406 Å.

Crystallite size was calculated using Scherrer Eqn., CS= Kλ/β cos θ, where CS is the crystallite size, constant K=0.94, β is the full width at half maximum (FWHM) (β=FWHM x π/180), λ=1.5406×10-10 and cos θ =Bragg angle (Pattanayak M and Nayak PL. 2013; Shobha G. *et al.*, 2014; Anandalakshmi K., *et al.*, 2016; B.D. 1978).

3.2.12.2. Scanning Electron Microscope (SEM) analysis

Scanning electron microscopy as (JSM- IT\500, Jeol, Boston., MA. USA), used for the examination of the gold, silver, and iron nanoparticles, and confirm their surface shape. The dry granules obtained from this green biosynthesis method by the steps in the aforementioned working methods, diluted with deionized water at a ratio of 10:1 g/ml. These dry granules were subjected to structural characterization by SEM analysis according to the (National Institute of Standards and Technology, NIST\2007; P. Prakasha. *et al.*, 2013; Fultz, B., & Howe, J. 2013). The utilization of FTIR spectroscopy (S\700,Nicolet,MA, USA) enabled the identification of the functional groups present on the iron surface and their participation in the biogenesis of iron nanoparticles (NIST. National Institute of Standards and Technology. 2022).

3.2.12.3. Fourier Transform Infra-red Spectrophotometric (FTIR) analysis

The mixture that was shaken by the magnetic stirrer for a period of (4 hours), and after the attraction of the iron nanoparticles (Fe NPs) around the metal capsule that rotates inside the beaker that was placed on the magnetic stirrer, was collected and analyzed by FTIR-spectroscopy. The functional groups of iron surface and were involved in the green biosynthesis of Fe-NPs were identified by using FTIR-spectroscopy (S\700, Nicolet, MA. USA) (B. Lesiak, *et al.*, 2019).

The proposal coating biomolecules attached to the (Au NPs) surface, responsible for its reduction and stabilization, are identified by FTIR (A. Dzimitrowicz, *et al.*, 2018; M. Montes, *et al.*, 2011). The sample is placed along the travel path of IR beam, in which the beam can penetrate through the sample and transmit to the detector (Y. Chen, *et al.*, 2015).

FTIR has been used for the nanoscale spectroscopic chemical identification of polymers, and for strain characterization and relaxation in crystalline materials (S. Bensmann, *et al.*, 2014). With the detection of phase, nano-FTIR provides complete information about near fields, which is essential for quantitative studies and many other applications. For example, for soft matter samples (organics, polymers, biomaterials, etc.), φ directly relates to the absorption in the sample material (T. Taubner, *et al.*, 2004; P. Carney, *et al.*, 2012). This permits a direct comparison of nano-FTIR spectra with conventional absorption spectra of the sample material (F. Huth, *et al.*, 2012).

3.2.13. Influence of Nanoparticles Against *Streptococcus mutans*

3.2.13.1. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Tests

An assessment was conducted to determine the (Minimum Inhibitory Concentration, Minimum Bactericidal Concentration) of AuNPs, AgNPs, and FeNPs, in order to calculate their antibacterial effectiveness. The MIC and MBC were determined by a dilution procedure. Subsequently, the impact on cell viability was evaluated within a 24-hour. The bacterial isolates were cultured in nutrient broth media and subjected to serial dilution using various concentrations of AuNPs, AgNPs, and FeNPs 200, 150, 125, 100, 75, 50 mg/ml respectively for 3-4 hours. After incubating the samples at 37°C for 24

hours, the bacterial concentration was measured by determining the optical density at 600nm, as described in the study by (Kumar *et al.* 2015).

The MIC was found by diluting the broth in experiments, whereas the MBC was calculated by identifying the lowest concentration of the antibacterial agent that reduces the viability of the original bacterial inoculum by a predefined amount, as adapted from (Krishnan *et al.* 2015).

3.2.13.2. Anti-bacterial activity of nano-materials on *S. mutans*

In this study, the usual inhibition diameters were measured as prescribed (Okoli and Iroegbu, 2004). The inoculums used in this study were set by addition *S. mutans* isolated colonies grown up on a nutrient agar plate to 5 ml of usual sterile saline matched to the standard McFarland tube (1.5 × 108 cells/ml). The sterile swab was castoff to extractinocula as of the suspension of the bacteria. These inocula were streaked on the MHA plate then left- hand to dry. AuNPs, AgNPs, and FeNPs (400, 200, 100, 50 mg/ml) were inserted into 4 wells, and permitted to position at room temperature for 1hr to disperse to medium before incubation at 37°C for 24hr. Zones of inhibition were assessed by a ruler and matched to the inhibition zones identified to determine the resistance or sensitivity of the organism to AuNPs, AgNPs, and FeNPs, respectively.

3.2.14. Ethical Approval

All participants involved in this study have been informed, and verbal consent will be provided by each one prior to sample collection.

SPSS statistical study performed in the results of this study.

Chapter Four

Results and Discussion

4.Results and Discussion.

4.1. Isolation and identification of bacterial isolate from dental caries

Thirty six *Streptococcus mutans* isolates were obtained from 100 clinical specimens of caries collected from both male and female patients with different ages. The morphological examination of isolates performed by streaking method on a modified (Mitis-Salivarius Agar Base) supplemented with (2 U/ml of Bacitracin and 2gm of 1% potassium tolerate), after incubation for 24 hours at 37°C. The bacterial colonies displayed characteristics of being large, *streptococcal* shape, low convex, blue in color, moist, and smooth. These characteristics are depicted in figure (4.1 A and B). Isolates were subsequently purified on the same agar media through sub-culture. (MacFaddin, 2000).

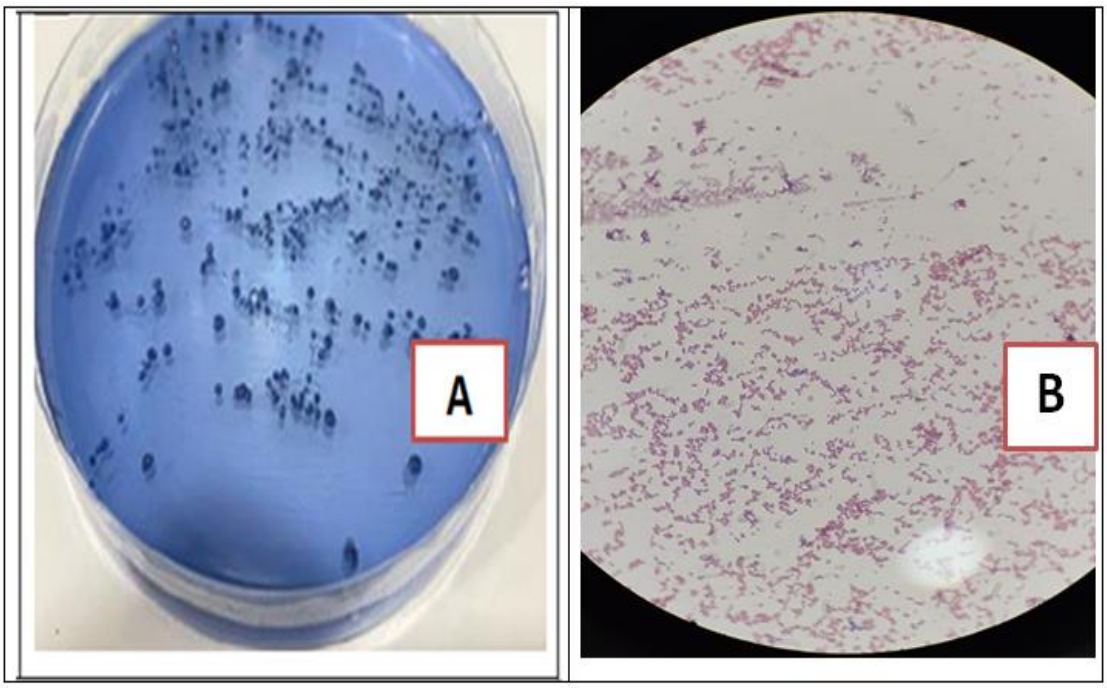


Figure (4.1): A The colonies of *Streptococcus mutans* were isolated on a modified (Mitis-Salivarius Agar Base) supplemented with (2 U/ml of Bacitracin and 2 gram of 1% potassium tolerate). **B** Isolates present on the slide were subjected to morphological analysis using light microscope (under 40X light microscope), following the implementation of the Gram Stain technique.

The present study employed a targeted primer, specifically the (*Sm479*) gene primer, to investigate the identification of *S. mutans*. The DNA extracted from the bacterial samples of *S. mutans* was subjected to agarose gel electrophoresis in order to identify the presence of the virulence gene *Sm479*. A total of 30 isolates showed a distinct band with a size of (479 base pairs bp) as shown in (figure 4:2) showed that all lines are a positive result completely, which is consistent with the results reported by (Zadeh, 2016).

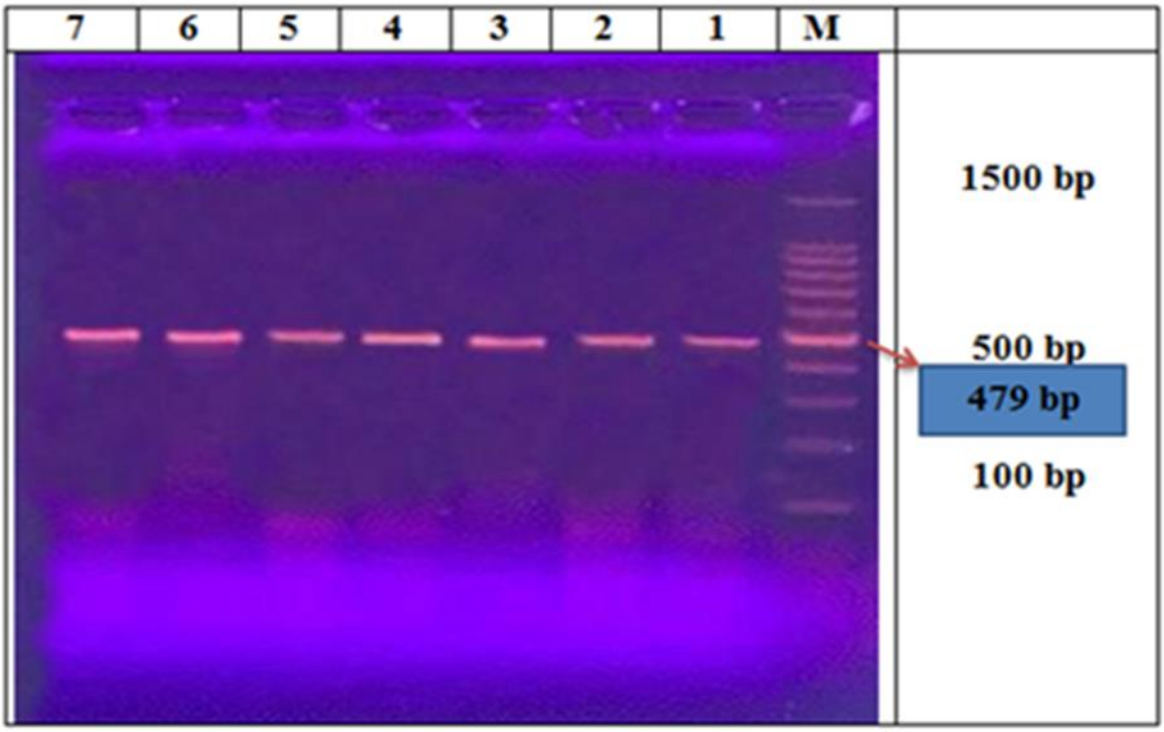


Figure 4.2: Agarose gel electrophoresis specific PCR product for 479bp, using 1.5 agarose gel at 90V for 1hr. in 1x TBE buffer, and visualization under transilluminator UV after staining-red safe. Lane L:bp DNA ladder. All lines shows positive result (479-bp fragment).

4.2. Biofilm Formation of *Streptococcus mutans*

The estimation of biofilm development on polymeric surfaces by *S. mutans* isolates was conducted using the microtiter plate crystal violet method, as described in the studies by (Zhou *et al.* 2018). Crystal violet is a negative dye that exhibits an affinity for positive molecules present on

the cell surface, as well as nucleic acids and polysaccharides, this provides a comprehensive assessment of the entire biofilm formation in gram positive, as described by (Djordjevic *et al.* 2002).

The result of biofilm formation grade recorded as OD values mentioned in the Appendix (2), and they were compared with ODc, these are estimated (13 non-adherence, 51 weak-adherence, 16 moderate-adherence, and 4 strong-adherence), about (13.68%, 53.68%, 16.84%, and 4.21%) respectively, as shown in figure (4.3).

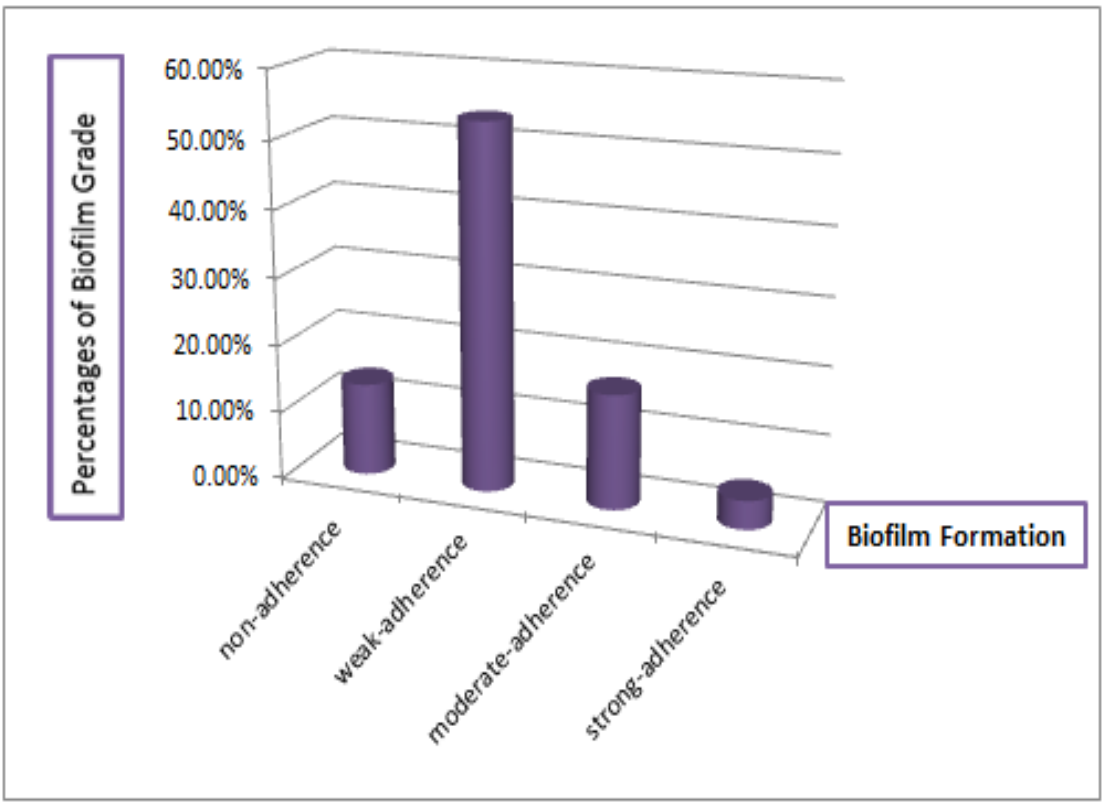


Figure 4.3: Biofilm formation grade of *Streptococcus mutans*.

Appropriating to the results in this study, the data that obtained, the emergence of strong, moderate, weak and non-production of biofilm refer to there was a significant difference in the ability to biofilm formation among the *S. mutans* isolates. So, there are differences in the ability of bacteria to adhere to the teeth and oral cavity, dependent on the ability of *S. mutans* on the biofilm formation, this can be explained by differences

in taking of medications by the patients, and the growth conditions such as ionic forces, pH and the number of subculture (Grivet *et al.*, 2000).

As biofilms are specifically microbial aggregates that rely on a solid surface and extracellular products, such as extracellular polymeric substances (EPSs) (Romero D., *et al.*, 2010). Bacteria move reversibly onto the surface,, and the bacteria multiply rapidly, resulting in the development of a mature biofilm. At this stage, the bacteria are stuck together, forming a barrier that can resist antibiotics and provide a source of systemic chronic infections. Thus, biofilms are a serious health threat (Huh AJ,. *et al.*, 2011; Hajipour MJ,. *et al.*, 2012).

Moreover, the bacteria within biofilms can produce super-antigens to evade the immune system. Therefore, despite the abundance of antimicrobial drugs, bacterial infections remain a major issue. The chronic infections related to planktonic bacteria and biofilms are always difficult to cure because of their inherent resistance to both antibiotics and host defenses.

4.3. Antibiotic Susceptibility Test (AST)

The compilation of antibiotic susceptibility testing lists was conducted by including data and thresholds from authoritative sources such as the Clinical Laboratory Standards Institute (CLSI, 2022), the European-Committee on Antibacterial Susceptibility-Testing (EUCAST), and the United States Food and Drug Administration (FDA).

Table 4.1: Antibiotic susceptibility test for *Streptococcus mutans*:

Antibiotic Group	Antibiotic	Symbol	Sensitivity No. (%)	Intermediate No. (%)	Resistant No. (%)
Cephalospoeins	Cefixime	CFM	12 (33.33%)	Non	24 (66.66%)
	Ceftazidime	CAZ	9 (25%)	3 (8.33%)	24 (66.66%)

	Ceftriaxone Cephalosporin	CRO CEP	Non Non	7 (19.44%) 9 (25%)	29 (80.55%) 27 (75%)
Macrolides	Erythromycin	E	Non	Non	36 (100%)
Penicillins	Piperacillin/tazobactam Rifampicin	PZT RA	16 (44.44%) 3 (8.33%)	12 (33.33%) 3 (8.33%)	8 (22.22%) 30 (83.33%)
Monobactam	Azithromycin	AZM-	3 (8.33%)	11 (30.55%)	22 (61.11%)
Tetracyclins	Doxycycline Tetracyclin	DA TE	Non 4 (11.11%)	7 (19.44%) 8 (22.22%)	29 (80.55%) 24 (66.66%)
Fluoroquinolones	Levofloxacin Ciprofloxacin Fluorinated ethylene propylene	LEV CIP FEP	Non 7 (19.44%) 3 (8.33%)	7 (19.44%) 2 (5.55%) 8 (22.22%)	29 (80.55%) 27 (75%) 25 (69.44%)
Bactrim	sulfamethoxazole	SXT	3 (8.33%)	3 (8.33%)	30 (83.33%)
Carbapenenems	Meropenem Imipenem	MEM IPM	5 (13.88%) 15 (41.66%)	4 (11.11%) 13 (36.11%)	27 (75%) 8 (22.22%)
Quinolone	Nalidixic acid	NA	Non	11 (30.55%)	25 (69.44%)

The data presented in (table 4.1) provides a comprehensive overview of the measurements of inhibition zone of bacterial growth, indicated the resistance, intermediate, and sensitivity exhibited by *S. mutans* towards a different types of antibiotics, as illustrated in the accompanying figure (4.4). The Multi-Drugs Resistant (MDR) of *S. mutans* to certain antibiotics in this study is documented between (15% to 100%), and these antibiotics can no longer be used to control or kill *S. mutans*. Resistance to multiple antibiotics (MDR) is mediated by a diverse array of mechanisms such as enzymatic mechanisms of drug modification, target

modification by mutations, enhanced efflux-pump expression and altered membrane permeability (Nikaido, H. 2009).

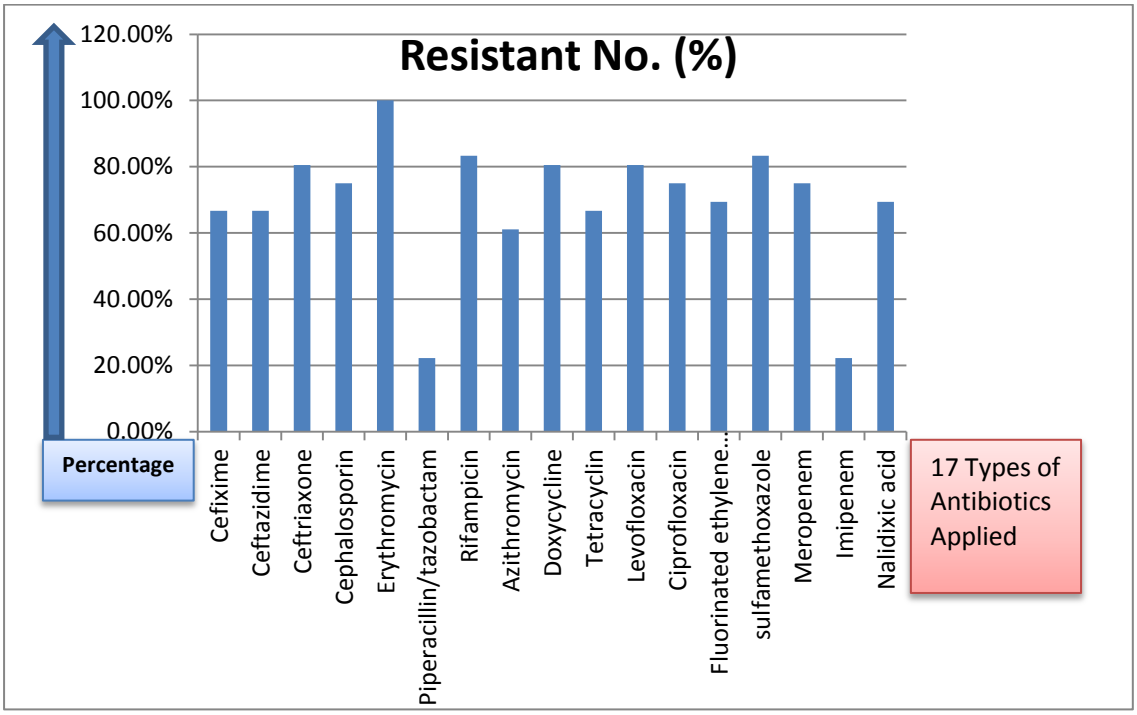


Figure 4.4: Percentages of resistance of *S. mutans* to types to 17 types of antibiotics.

Antibiotic resistance is a major subset of AMR, that applies specifically to bacteria that become resistant to antibiotics. Resistance in bacteria can arise naturally by genetic mutation, or by one species acquiring resistance from another. Resistance can appear spontaneously because of random mutations, but also arises through spreading of resistant genes through horizontal gene transfer. However, extended use of antibiotics appears to encourage selection for mutations which can render antibiotics ineffective (Dabour R. *et al.*, 2016).

Antimicrobial resistance (AMR) occurs when microbes evolve mechanisms that protect them from the effects of antimicrobials (drugs used to treat infections). All classes of microbes can evolve resistance where the drugs are no longer effective. Fungi evolve antifungal resistance, viruses evolve antiviral resistance, protozoa evolve

antiprotozoal resistance, and bacteria evolve antibiotic resistance. Together all of these come under the umbrella of antimicrobial resistance. Microbes resistant to multiple antimicrobials are called multidrug resistant (MDR) and are sometimes referred to as superbugs. Although antimicrobial resistance is a naturally occurring process, it is often the result of improper usage of the drugs and management of the infections (Saha M. and Sarkar A. 2021).

Antimicrobial resistance (AMR) is one of the top global public health and development threats. It is estimated that bacterial AMR was directly responsible for 1.27 million global deaths in 2019 and contributed to 4.95 million deaths (Antimicrobial Resistance Collaborators. 2022).

Antibiotic resistance is a major subset of Antimicrobial Resistance (AMR), that applies specifically to bacteria that become resistant to antibiotics (World Health Organization, 2014). Resistance in *S. mutans* can arise naturally by genetic mutation, or by one species acquiring resistance from another. Resistance can appear spontaneously because of random mutations, but also arises through spreading of resistant genes through horizontal gene transfer. However, extended use of antibiotics appears to encourage selection for mutations which can render antibiotics ineffective (Dabour R., *et al.*, 2014).

4.4.Biosynthesis of Nanomaterials

4.4.1.Biosynthesis of Iron Nanoparticles (FeNPs)

Iron nano-particles (FeNPs) were derived from biological source due to their ubiquitous presence in various living organisms, instead of extracting them from their mineral bulk, which can regenerate and revert to a solid state, a biological synthesis approach of FeNPs were achieved as a crude that present in oyster shell.

This way ensures the production of nanoparticles with suitable size and round shape, crucial for their intended function as antibiotics or antibacterial agent affected on *S. mutans* cell wall, cytoplasmic organelles rupture, or its DNA damaged.

Irregularly shaped nanoparticles are unable to effectively penetrate cell walls, rendering them ineffective as antibiotics and potentially causing cytotoxicity. FeNPs biological synthesis from oyster shell as a representative biological source, because it contains a high concentration of iron (Stir Bars., 2013). During the synthesis process, iron nanoparticles are produced with a notable efficacy as an antibiotic, devoid of any harmful properties.

The methodology for the biogenesis of iron nanoparticles (FeNPs) involves a series of steps, namely collection, cleaning, disinfection, breakdown, and grinding of oyster shell as shown in figure (4.5), to create a powdered form.

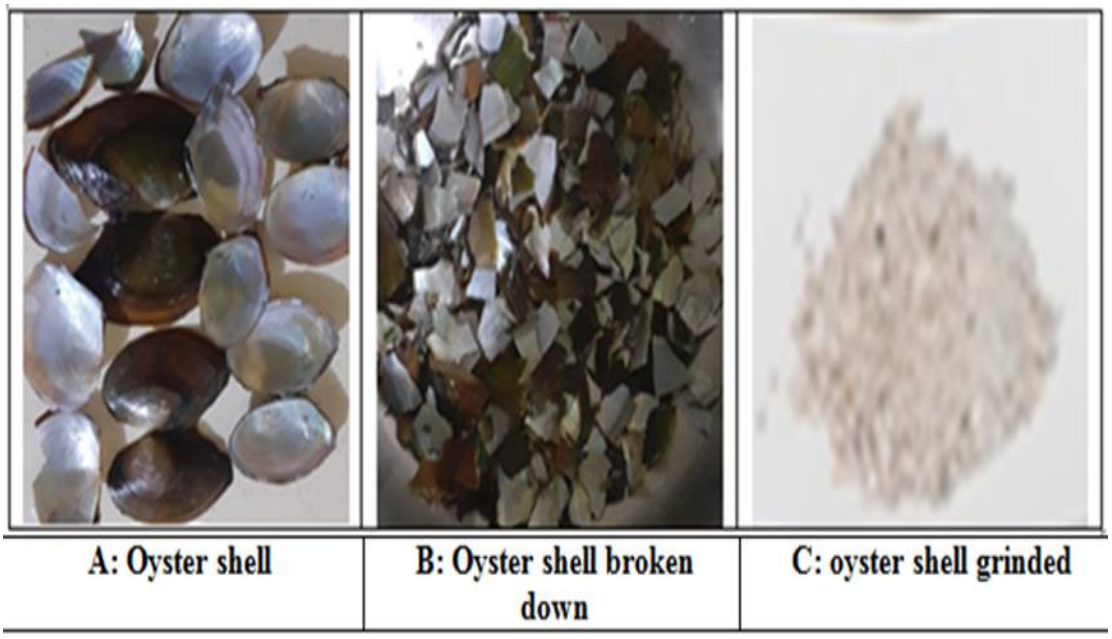


Figure 4.5: Oyster shell as a raw material for Fe-NPs biosynthesis.

De-ionized water was employed to immerse the powder, as it yielded a higher quantity of pure iron nanoparticles compared to the utilization of regular water. Hydrochloric acid (HCl) is classified as a potent inorganic acid, widely employed in various industrial applications. One notable use involves its role in the separation and purification of minerals, where it effectively disrupts the chemical bonds that hold them together.

The purpose of employing the magnetic stirrer device in this study is to facilitate the-aggregation of the iron-containing colloidal solution, hence enhancing the efficiency of the subsequent filtration process aimed at concentrating the raw material. The biogenesis process of iron nanoparticles involves the utilization of 10% solution of sodium hydroxide (NaOH) to effectively dissolve any undesired solid substances present in the raw materials employed for this synthesis procedure.

The utilization of a magnetic stirrer for the reason of agitating the mixture for a duration of approximately four hours enhances the process of extracting iron nanoparticles (FeNPs). This is achieved by the magnetic attraction of the FeNPs to the metal capsule, which functions as a conduit within the magnetic stirrer device, as shown in figure (4.6) A and B. An inverse correlation has observed between the initial (FeNPs) concentration and an adsorption capacity in the process (Perez-Aguilar NV., 2011).

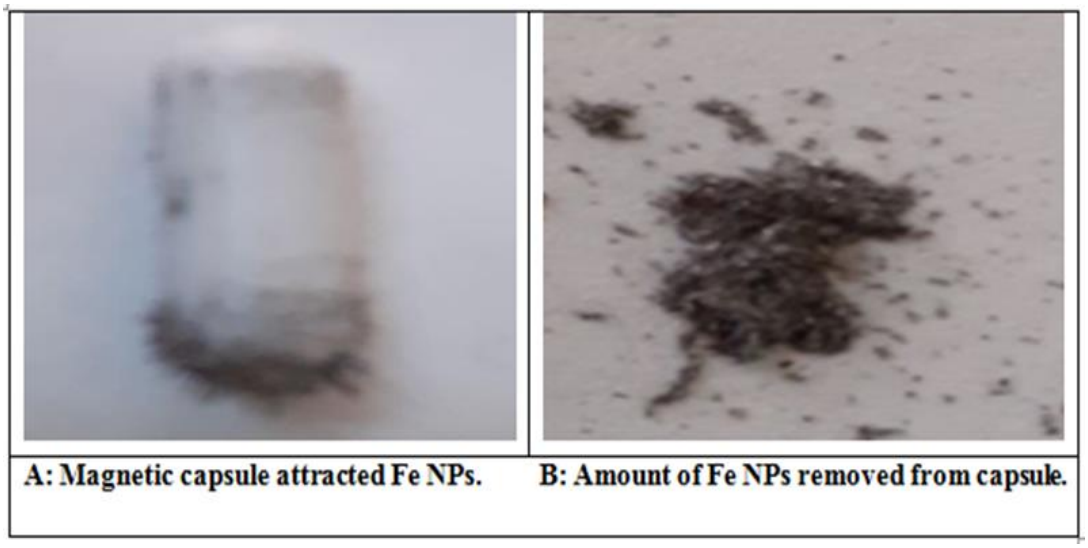


Figure 4.6: FeNPs created and afterwards localized in close proximity to the magnetic capsule.

4.4.2. Characterization of iron nanoparticles (Fe NPs)

4.4.2.1 X-Ray-Diffraction (XRD)

The iron nanoparticles (FeNPs) synthesized using green methods and extracted through biological processes were analyzed using X-ray determination (XRD-analysis). This analysis aimed to determination of the Fe-NPs nature, and obtain information about their average size, as described in reference (Joseph., 2003) and depicted in figure (4.7). X-ray diffraction (XRD) is a widely employed analytical-technique utilized to obtained of elemental-analysis, and chemical-characterization to material that given.

The process is contingent upon the interaction between a sources of X ray excitation and a given specimen. The properties-capabilities of this phenomenon can be attributed primarily to the fundamental premise that every element possesses a distinct atomic structure, resulting in a unique arrangement of peaks on its electromagnetic emission spectrum (Shobha G. *et al.*, 2013).

The use of the Scherrer Equation, as depicted in figure (4.8) was to calculate the average of FeNPs size by analyze of the peak amount in X-ray diffraction (XRD) picture.

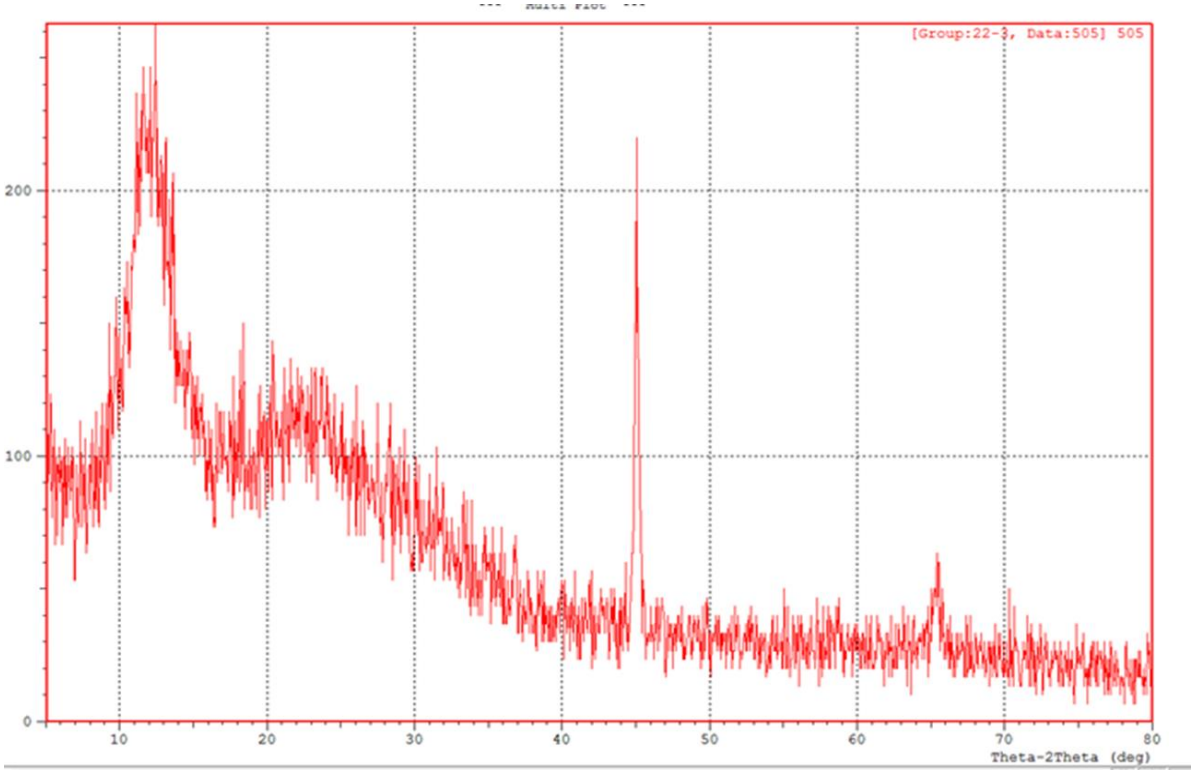


Figure (4.7): XRD analysis of iron nanoparticles (FeNPs) Synthesized.

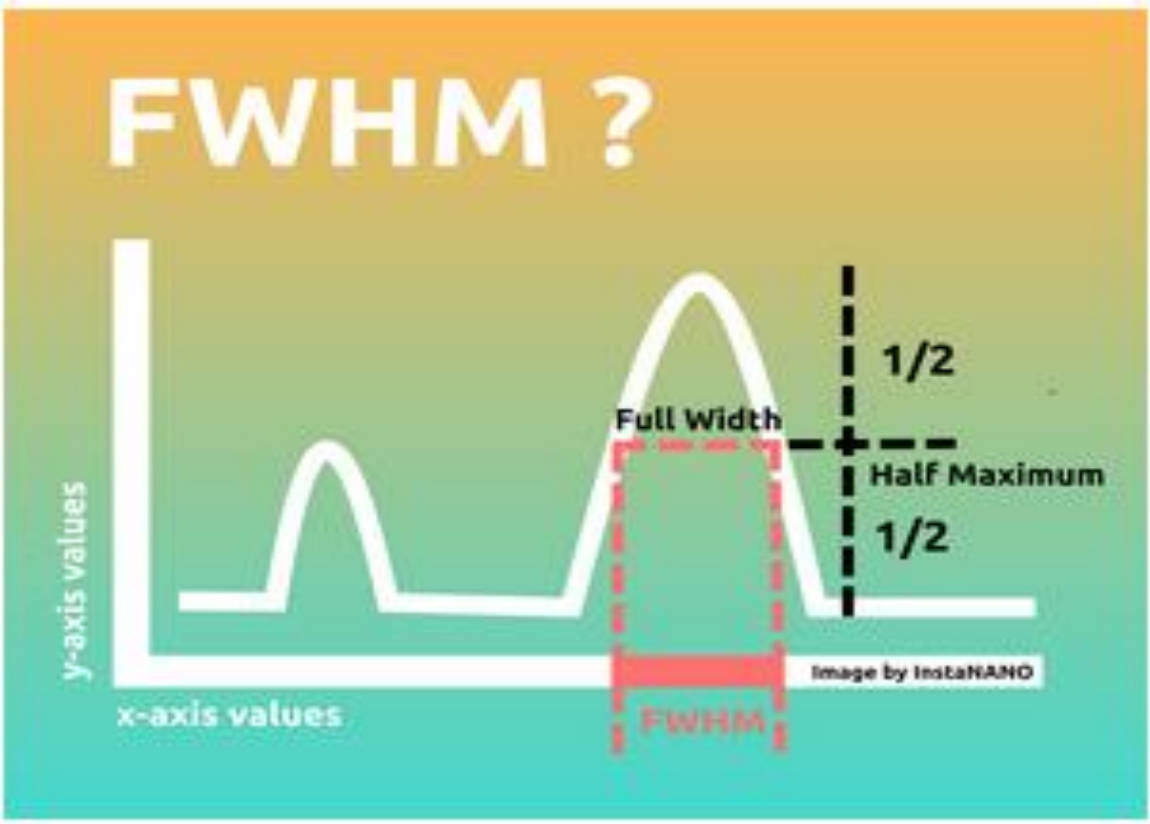


Figure (4.8):XRD Crystallite (grain) Iron nanoparticle size Calculator (Scherrer Equation). The result of applied Scherrer Equation is equal to (69.37 n.m), in the link https://instanano.com/all/characterization/xrd/crystallite-size

4.4.2.2 Scanning Electron Microscope (SEM) analysis of Fe NPs.

FeNPs spherical shape was observed using scanning electron microscopy SEM analysis with (45-100 n.m). In the context of (SEM) as shown in figure (4.9), it is typically necessary for a specimen to be thoroughly dried prior to analysis, as the specimen chamber operates under conditions of high vacuum. In order to analyze hard, desiccated substances like shells and other biological sources, minimal additional processing is necessary. However, the examination of living cells, tissues, and intact creatures with soft bodies necessitates chemical fixation to maintain and stabilize their structural integrity (Joseph., 2003). For the normal distribution, these would be the sample mean and the sample standard deviation. The number of particles needed for high accuracy

estimates of the average diameter is known to depend on the spread of the particle size distribution.

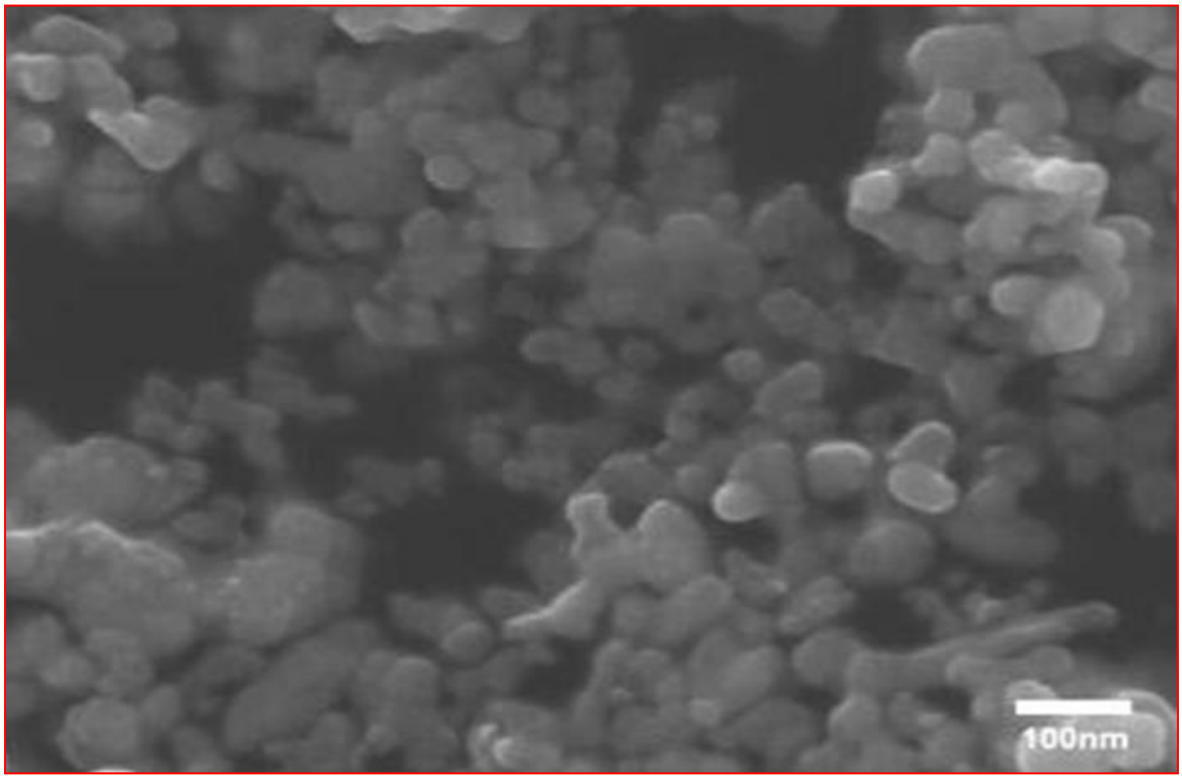


Figure (4.9): (SEM)-image of the (FeNPs) synthesized.

Table (4.2): The size of iron nanoparticles (Fe-NPs) was determined using (SEM):

Diameter of (FeNPs) n.m .
70.22
67.53
84.36
73.65
34.72
52.58
82.35
46.73

It has become clear by measuring the diameters of the iron nanoparticles resulting from the SEM analysis shown in table (4.2) that there are no significant differences in the sizes, there is normality in their sizes and within the natural nanoparticle limits that can play their role in penetrating the bacterial cell wall.

4.4.2.3. Fourier Transform Infra-Red Spectrophotometric (FTIR) Analysis of FeNPs.

This method is employed to acquire an infrared spectrum that represents the absorption or emission of a substance in solid, liquid, or gaseous form. The Fourier spectrometer is capable of collecting spectral data with great resolution across a broad range of wavelengths simultaneously. Dispersion spectrometers, that concurrently measure intensities within a limited range of wavelengths (P. Griffiths, *et al.*, 2019), are comparatively disadvantaged in relation to this method. FTIR-spectroscopy employed to detect the localization of functional groups accountable for the creation of iron nanoparticles (Fe NPs) derived from an oyster shell. Figure 4.10 depicts the absorbance spectra of iron nanoparticles (Fe NPs) within the wave area spanning from 1000 to 3500 cm-1. Two distinct peaks were identified at 1636.34 and 3282.19 cm^{-1}, indicating characteristic features.

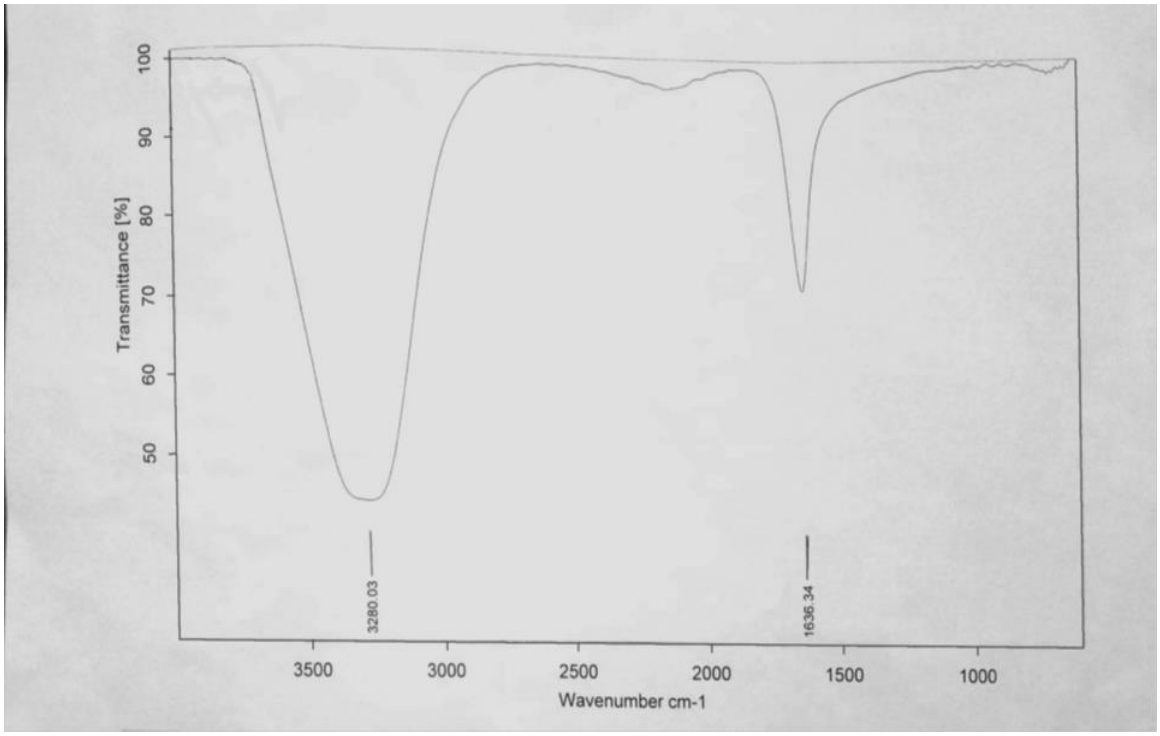


Figure (4.10): FTIR spectra to show the (Fe-NPs) synthesized peaks.

4.4.3. Biosynthesis of Gold Nanoparticles (AuNPs)

Microorganisms can act as a potential factory for gold nanoparticles production (Ghosh S. *et al.*, 2021). The biosynthesis mechanism was found to be both extracellular and intracellular for bacteria according to the location of (Au NPs) production (Abbasi T. *et al.*, 2015; Lee K.X. *et al.*, 2020). However, the extracellular synthesis of gold nanoparticles is the most common (Lee K.X. *et al.*, 2020). Gold ions are first trapped on the surface or inside the microbial cells and then reduced to nanoparticles in the presence of enzymes (Ghosh S. *et al.*, 2021).

The attractive procedure is using microorganisms such as bacteria to synthesize gold nanoparticles (Au NPs) recently. An earlier study found that *Bacillus subtilis* (Ghosh S. *et al.*, 2021) were able to reduce Au^{3+} ions to gold nanoparticles with a size range of 5–25 nm inside the cell walls.

In the process of AuNPs biosynthesis, it is necessary to activate bacteria in order to ensure the presence of metabolic material in both

extracellular and intracellular locations (Lee K.X. *et al.,* 2020). This activation is crucial for achieving high-quality Au NPs. The stock strain of *Bacillus subtilis* was grown by introducing it into a 10 ml quantity of Brain Heart Infusion Broth (BHI). The culture was then kept at (37 °C for 24 hrs), resulting in the production of 24-hour-old bacterial-cells. The synthesis of gold nanoparticles was observed in the extracellular cell supernatant of the bacteria, as demonstrated in this work. From the application standpoint, this presents a significant advantage compared to an intracellular synthesis process.

The extracellular synthesis of gold nanoparticles (Au NPs) is a well observed phenomenon. In this process, Au NPs are initially captured either on the surface or within the microbial cells. Subsequently, enzymatic activity facilitates the reduction of these captured Au NPs, this step leads to the synthesis of nanoparticles. The selection of *Bacillus subtilis* as the preferred organism for the production of gold nanoparticles (Au NPs) was a deliberate select based on it's shown capability to efficiently achieve the desired target (Ghosh S. *et al.*, 2021).

The bacteria demonstrates favorable attributes for the reduction of Au3+ ions within its cellular membranes, leading to create of spherical Au nanoparticles with a size distribution ranging from 5 to 25 n.m. To enhance the synthesis of nanoparticles, it's important to inject a re-inoculum of *Bacillus subtilis* in to a 1000-ml volume of Brain Heart Infusion (BHI) media. The re-inoculum is acquired through the addition of 1 ml of active bacteria to the medium. The resultant mixture is thereafter subjected to incubation at a temperature of 37 degrees Celsius for a period ranging from 48 to 72 hours.

The incubation period facilitates the bacteria's ability to undergo sorting and generate primary and secondary metabolites, which play a crucial role in the production of nanoparticles. The process of centrifugation is essential for the purpose of isolating the supernatant components from other substances, specifically the secondary metabolic materials. The $AuCl_4$ solution is commonly employed in conjunction with *Bacillus subtilis* for the purpose of decreasing AuNPs (Wang, D. *et al.*, 2017).

The inclusion of sodium hydroxide NaOH in the experimental procedure is a crucial step aimed at preventing the aggregation of gold nanoparticles AuNPs in the bottom of the beaker. Additionally, it facilitates the attainment of a homogeneous solution under specific conditions, namely a pH of 9 and a temperature of 37°C. The sample was protected from oxidation by light using silicone covering. It was then placed on a magnetic stirrer device operating at 100 rpm for a duration of 24 hours at a temperature of 20°C. This procedure was carried out to facilitate the reduced of AuNPs.

4.4.4 Characterization of gold nanoparticles (Au NPs).

4. 4.4.1. X Ray Diffraction (XRD.) of (Au NPs).

XRD analysis was employed to provide evidence for the existence of elemental gold within the supernatants of the bacterial culture. The pre-treatment method was employed to provide enhanced resolution of the Bragg peaks. Subsequently, every powder specimen underwent analysis utilizing a Philips automated X-ray diffractometer equipped with a Philips PW 1830 X-ray generator. The resulting diffracted intensities were acquired between the angular range of 30º to 80º at an increment of 2ºθ (D.V. Leff, *et al.*, 1996), as shown in figure (4.11). Crystals exhibit a periodic arrangement of atoms, while X-rays can be conceptualised as

electromagnetic waves. Similar to the occurrence of secondary circular waves originating from a lighthouse when struck by an ocean wave, the impact of an X-ray on an electron results in the generation of secondary spherical waves originating from the electron. Based on the Paul Scherrer equation, the determined size of the gold nanoparticles is 10.69 n.m.

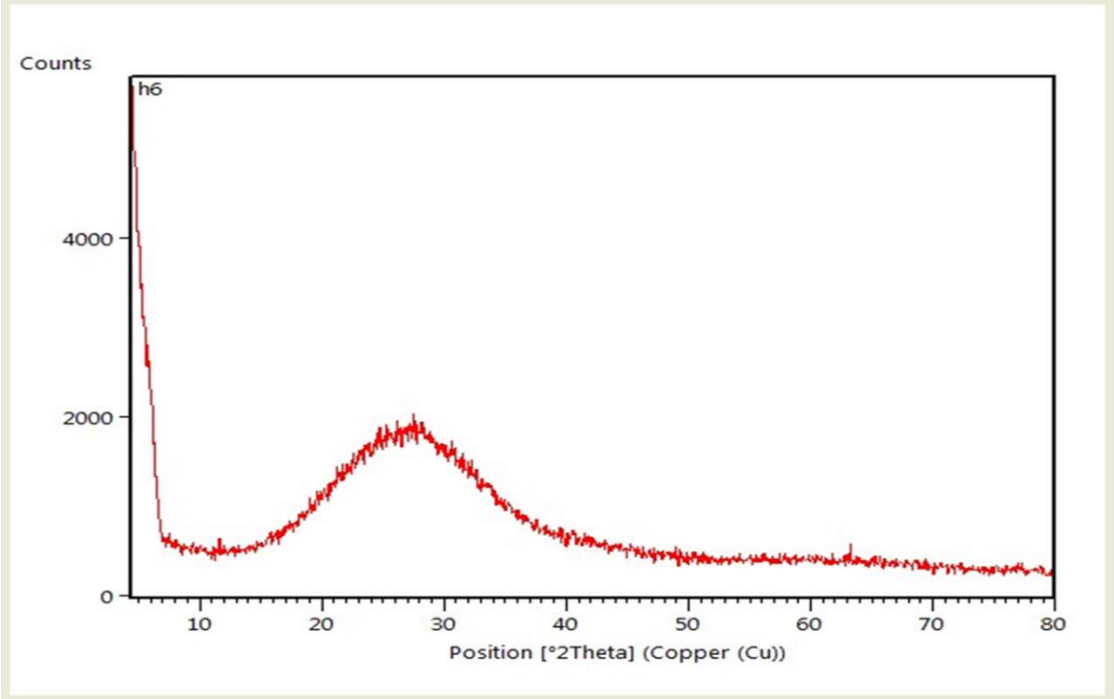


Figure (4.11): X-ray diffraction (XRD) of AuNPs.

4.4.4.2 SEM analyzed of (Au NPs)

A superior electron probe is produced by concentrating electrons discharged from an electron source, referred to as the electron cannon, onto the specimen's surface through a sequence of electro optical lens components. The resultant spot size spans a range of dimensions, varying from a few angstroms to several hundred nanometers. The current scanning electron microscopy (SEM) equipment has mechanical functionalities that enhance the use of various imaging and detection methods, allowing for meticulous analysis of several aspects of sample composition.

In comparison to traditional light microscopy, scanning electron microscopy (SEM) produces images by the detection and storage of various signals resulting from the interactions between an electron beam and the sample. The methodology utilised in this research enables the fabrication of environmentally friendly gold nanoparticles (Au NPs) possessing distinct form and size attributes (Fultz, & Howe, 2013). These aforementioned properties fall within the requisite range for proficiently infiltrating the cellular walls and cytoplasmic constituents of bacteria. The particles demonstrate a spherical morphology and possess dimensions that are suitable for their intended biological utilization., as shown in figure (4.12), and table (4.3).

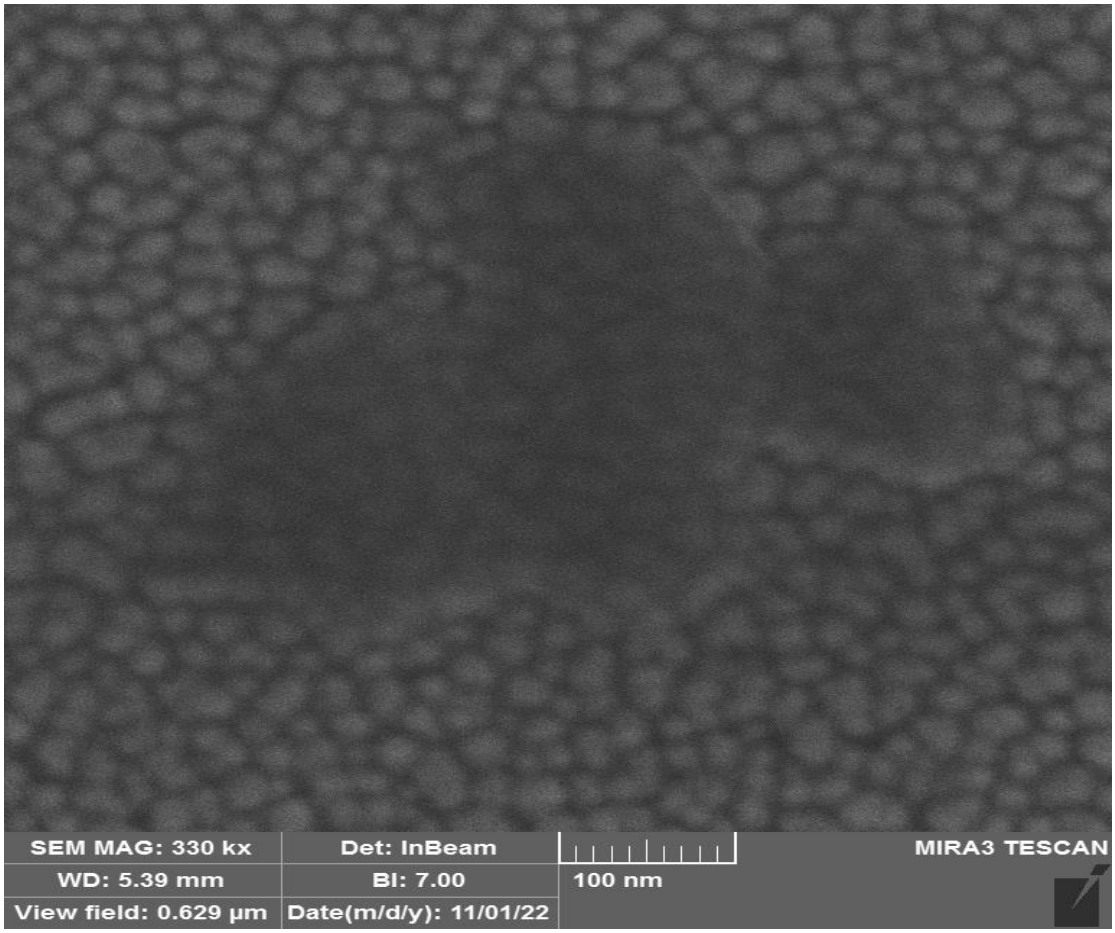


Figure (4.12): A scanning electron microscopy (SEM) image depicting the synthesized Gold nanoparticles (Au-NPs).

Table (4.3): The size of (Au NPs) is determined , when using SEM:

Diameter of (AuNPs) n.m .
54.83
46.27
67.23
72.46
32.54
58.27
47.63
62.18

4.4.4.3 Fourier transform infra-red spectrophotometric (FTIR) analysis of (Au NPs).

The gold nanoparticles (Au NPs) produced through the action of *Bacillus subtilis* were examined using (FTIR) analysis in order to determine the biomolecules responsible for stabilising the nanoparticles in a liquid medium (Shankar S. *et al.*, 2003). The gold nanoparticles (Au NPs) produced by *Bacillus subtilis* exhibited prominent peaks at 660.68, 996.97, 1053.66, 1412.61, 1558.06, 1637.90, and 3435.79 cm−1, as depicted in figure 4.13.

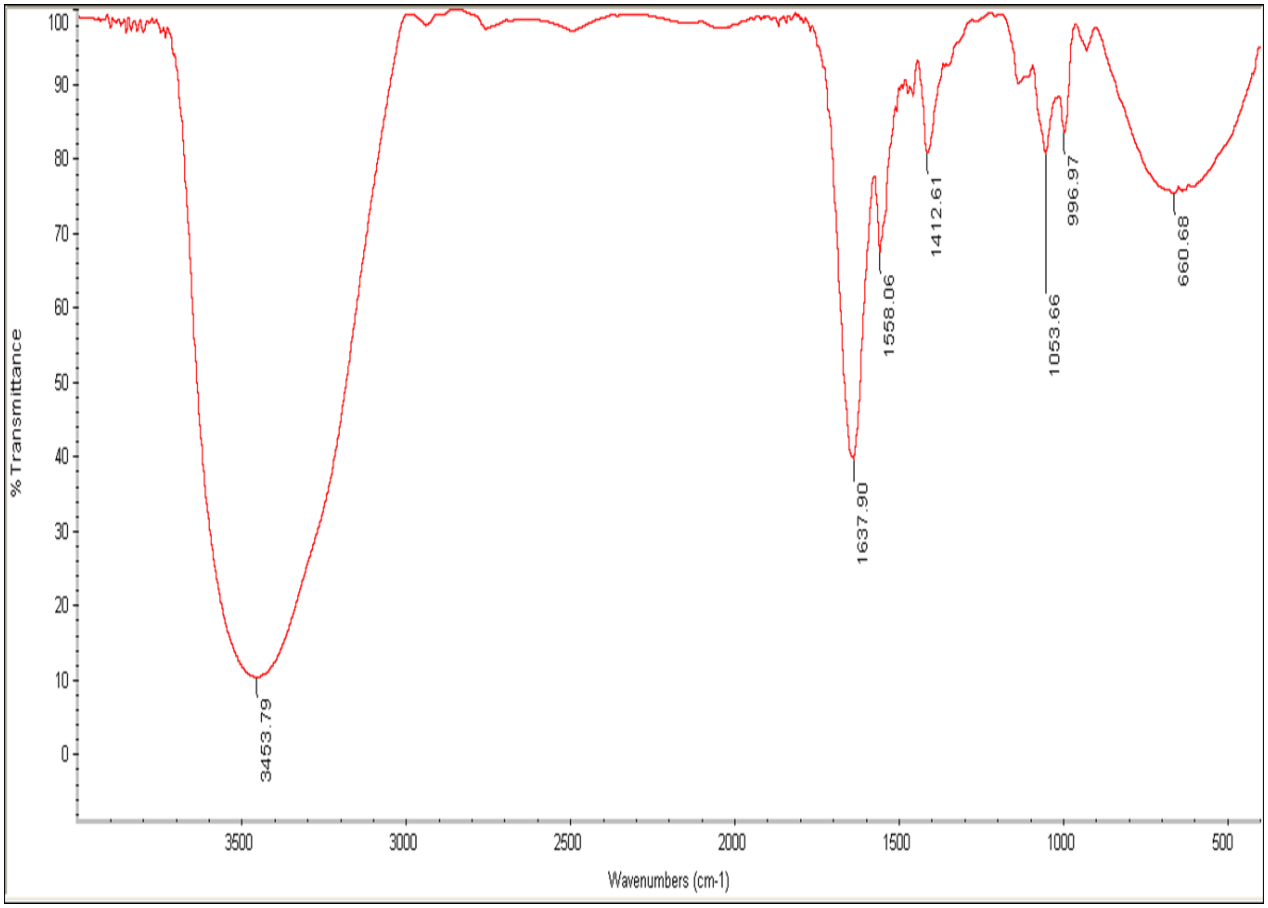


Figure (4.13): FTIR-spectra showing the characteristic peak's associated with the creation of (Au-NPs).

4.4.5 Biosynthesis of Silver Nanoparticles AgNPs.

Biosynthesis of silver nanoparticles using microorganisms has found interest recently since last decade because of their prospect to synthesize nanoparticles of various size, shape and morphology which are eco-friendly. Here, an eco-friendly method for production of silver nanoparticles from *Bacillus clausii* (Koel Mukherjee., *et al.*, 2018).

High gravity conditions generated by centrifuge are applied in the chemical industry, casting, and material synthesis (Yin, Xi., et al., 2010; Mesquita, R.A. et al., 2007).

In this study, a sustainable approach for biosynthesized of AgNPs using *Bacillus-clausii* is presented as the study of (Koel Mukherjee. *et al.*,

2018). The production of antimicrobial compounds with activity against Gram-positive bacteria, such as *Streptococcus mutans* has been observed (Efficiation, 2020). *Bacillus subtilis* exhibits robust antibiotic resistance, so conferring a therapeutic advantage in the restoration of the normal microbial community, particularly in the context of antibiotic administration and subsequent periods. The *Bacillus-clausii* strain was cultivated in brain heart broth and thereafter incubation at 37 °C and 48-72 hrs. This cultivation process aimed to get secondary metabolites. To separate the components, centrifugation was performed at a speed of 500 rpm for a period of 20 minutes. This separation technique finds use in various industries such as the chemical industry, casting, and material synthesis (Yin, Xi. *et al.*, 2016; Mesquita, R.A. *et al.*, 2007).

The supernatant contains secondary metabolites that engage with the chemical employed in the synthesis of silver nanoparticles (Ag NPs). A supernatant solution consisting of 600 ml was subjected to the addition of 10 mg of AgNO3. The resulting mixture was then placed on a magnetic stirrer device, where a stir bar was utilised to facilitate the stirring and mixing process of the solution at a speed of 145 rotations per minute. The utilisation of a shaker apparatus at a temperature of 37°C for a duration of around 1-2 days resulted in a discernible alteration in color, namely at a PH=9.

4.4.6 Characterisation of (Ag NPs).

4.4.6.1 XRD of Ag NPs.

The X-ray diffraction pattern of the silver nanoparticles (Ag NPs) that were synthesized using *Bacillus clausii* illustrated in figure (4.14). The crystalline structure of the silver nanoparticles (Ag NPs) was further confirmed by the utilisation of X-ray diffraction (XRD) analysis. The X-

ray diffraction (XRD) pattern of the nanoparticles was analysed, which were derived from the dried colloid samples. X-ray diffraction (XRD) is an instrumental technique that provides useful insights into the atomic structure of many materials. X-ray diffraction (XRD) is frequently utilised not only for the qualitative determination of minerals, but also for the quantitative assessment of mineral data (Said S. *et al.*, 2007).

XRD is a highly useful technique for characterizing materials, particularly in the context of proving the production of silver nanoparticles (AgNPs), determining their crystal structure, and calculating the size of these crystalline nanoparticles (Daizy Philip. 2011; Sumitra Chanda. 2013). Based on the Paul Scherrer equation (Bernal JD, 1924), the determined size of the silver nanoparticle (AgNPs) is equal to (43.18 n.m).

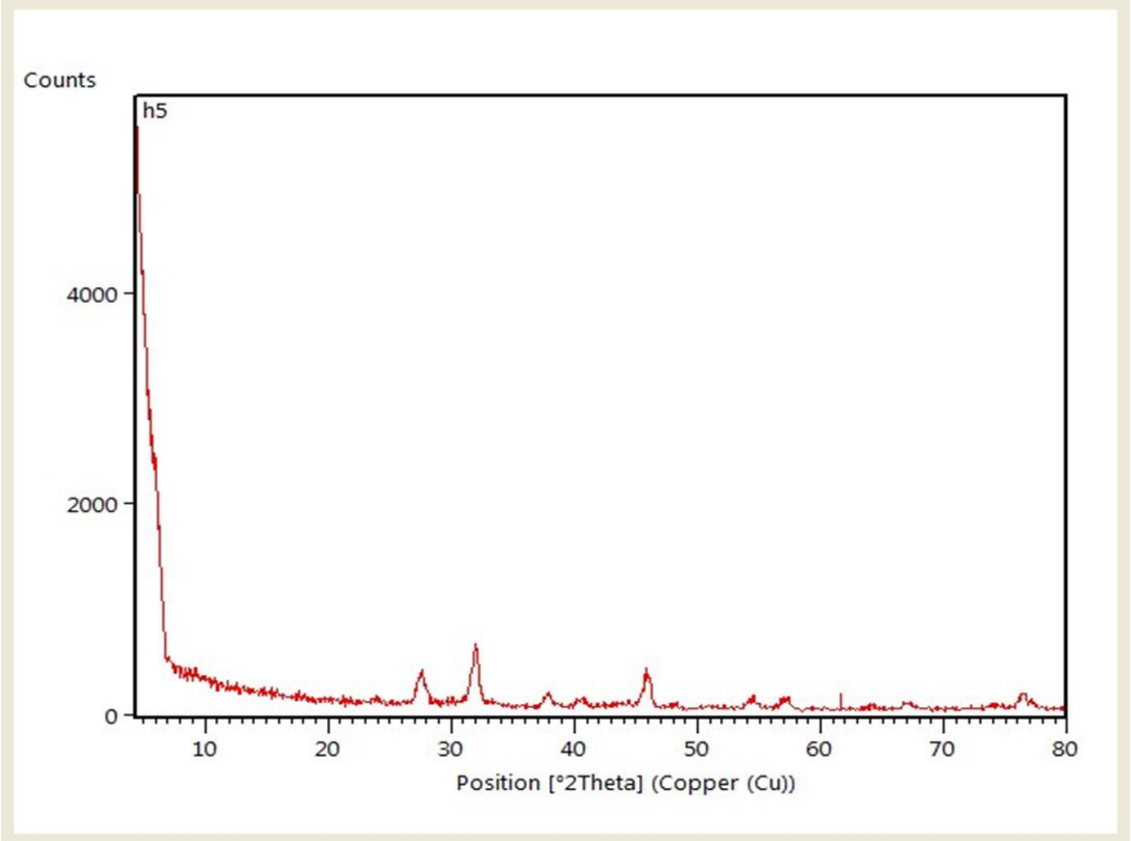


Figure (4.14): XRD analysis of Silver nanoparticles (Ag-NPs). synthesized.

The distinctiveness of the peak provides unambiguous evidence that the particles are within the nanoscale range. The presence of macromolecules in *Bacillus clausii* likely contributed to the observed peak broadening and noise, potentially leading to the decrease of silver ions. Therefore, X-ray diffraction (XRD) unequivocally demonstrates that the silver nanoparticles (Ag NPs) synthesized in this study possess a crystalline structure. The observation of broadened Bragg's peaks at their lower regions suggests the presence of silver nanoparticles of reduced size (Smitha S L. *et. al.,* 2009; P. Gurusamy, and K. Mahendran, 2013). The presence of certain bioorganic chemicals or proteins in *Bacillus clausii* may account for the observation of several unassigned peaks, which subsequently crystallize on the surface of the silver.

4.4.6.2 Scanning Electron Microscope (SEM) analysis of (Ag NPs).

The particle size measurements were obtained using the scanning electron microscopy (SEM) technique. The pictures were captured using a JEOL-JEM 6390 microscope from Japan, operating at a magnifications of (40, 000×) and a voltage of 20.00 kV. The recorded particle sizes ranged between 30 and 90 n.m, as depicted in figure (4.15). The X-ray patterns were subsequently acquired within the temperature range of 20-80 ◦C (Prakasha, 2013) The author proceeded to elaborate on the diverse detection modalities, potentialities, and theoretical aspects of scanning electron microscopy (SEM).

Additionally, the author detailed the development of the initial high-resolution SEM, and subsequent advancements were documented by the research team led by Zworykin (Zworykin. *et. al.,* 1942). One potential use is to the quantification of the surface roughness of ice crystals. The proposed approach involves the integration of variable-pressure

environmental scanning electron microscopy (SEM) with the three-dimensional (3D) capabilities of SEM in order to quantify the roughness shown by individual ice crystal faces. Subsequently, this roughness data is transformed into a computer model, enabling application of further statistical analyses to the model. (Butterfield. *et. al.*, 2017).

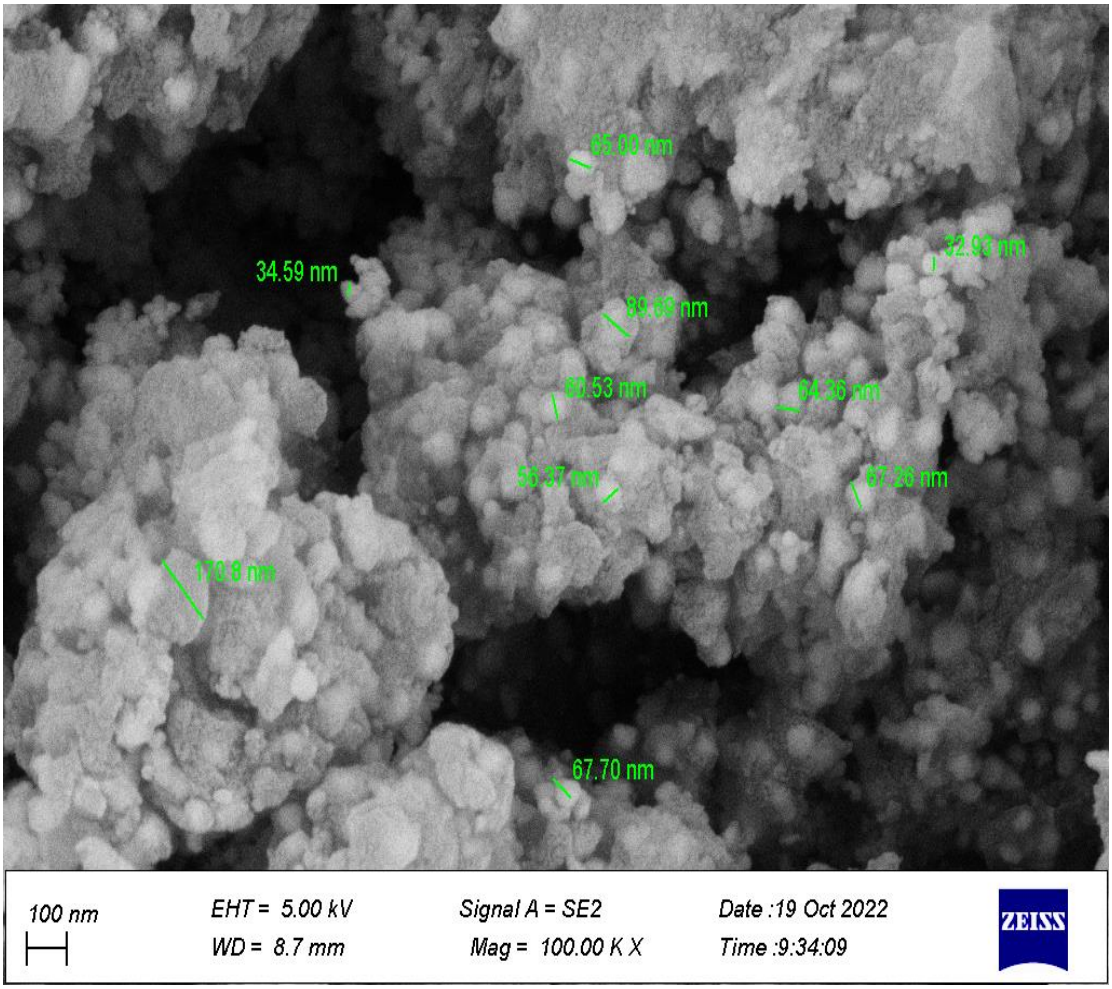


Figure (4.15):(SEM) of Silver nanoparticles (Ag-NPs). synthesized.

Table (4.4): The size of Silver nanoparticles (Ag NPs) is determined , when using SEM:

Diameter of (Ag NPs) n.m.
65.00
32.93

89.69
64.36
60.53
67.26
56.37
67.70

4.4.6.3 Fourier transform infra-red spectrophotometric (FTIR) analysis of (Ag NPs)

FTIR is a widely employed method for analysis of infrared absorption and emission spectra. It is a commonly employed technique to investigation of Ag-NPs. In this study, the utilization of FTIR was to detect Ag NPs by analyzing the bond characteristics associated with a wide range of nanoparticles. This enabled us to perform quantitative analysis of Ag NPs with the assistance of FTIR (Taubner T. *et al.*, 2004; Carney PS. *et al.*, 2012). The utilization of Fourier Transform Infrared Spectroscopy (FTIR) is evident in figure (4.17), where the depicted functional groups span a range of wavenumber between 500 and 3500 cm-1. The functional-groups are characterized by the wavenumbers (463,10 , 619,67 , 648,95 , 1046, 95 , 1099,39 , 1423,20 , and 1576,51cm $^{-1}$).

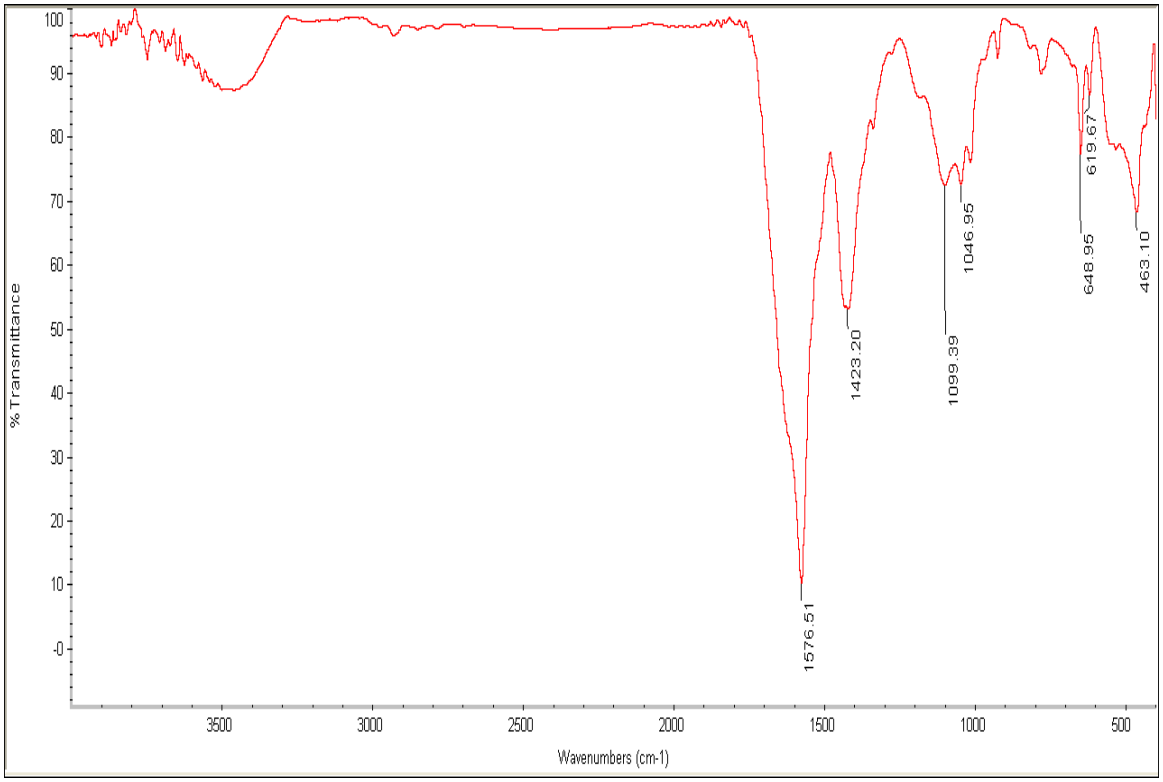


Figure (4.16): The FTIR shows the characteristic peaks associated with the creation of AgNPs.

Studies have shown that many NPs can prevent or overcome biofilm formation, including Au-based NPs (Yu Q., *et al.*, 2016), Ag-based NPs (Markowska K., *et al.*, 2013), Fe3O4 NPs (Chifiriuc C,. *et al.*, 2012). Greater prevention of biofilms is achieved by a smaller size and higher surface area-to-mass ratio, and the particle shape of NPs also has a remarkable effect on biofilm destruction (eg, NPs with a rod like shape are more effective than NPs with a spherical shape).

In this study, the shape of all types of nanoparticles is round, and the average particle sizes of nanoparticles (FeNPs, AuNPs, and AgNPs), as shown by the Scherer Equation results, are: (69.37 nm, 10.69 nm, and 43.18 nm) respectively, appropriated with that shown in (SEM) technique of each ones. There is normality (no significant differences) in the distrebution of each type of them, as shown in figure (4.17).

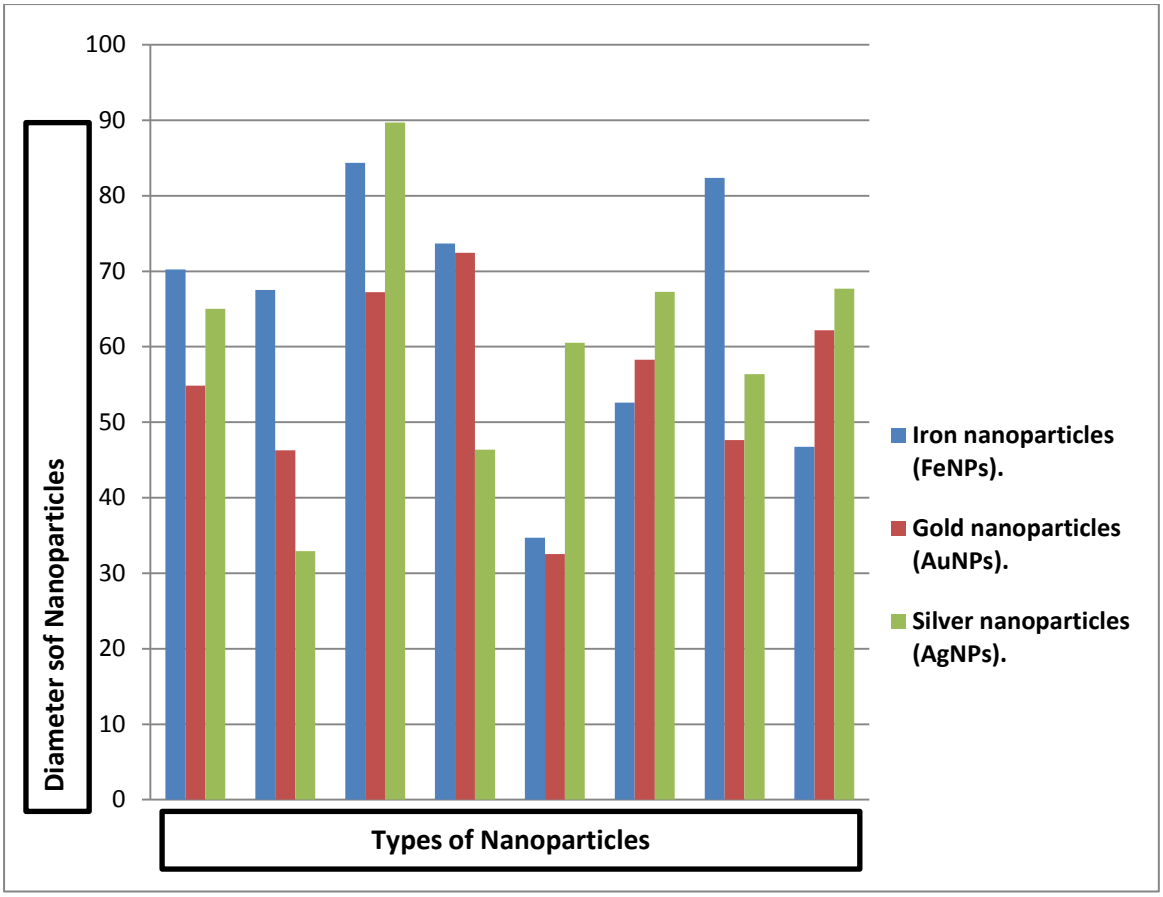


Figure (4.17): The range of particle size of (FeNPs, AuNPs, and AgNPs).

4.7. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) tests

The minimum inhibitory concentration for AuNPs, FeNPs, and AgNPs is 100, 125, and 75 mg/ml, respectively. While it shows in each figures (4.18, 4.19, and 4.20) that the minimum lethal concentration for AuNPs, FeNPs, and AgNPs was 125, 150, and 100 mg/ml, respectively, as shown in table (4.5).

Table (4.5): (MIC), and (MBC) of AuNPs, FeNPs, and AgNPs against *S. mutans*.

Nano-materials	**Minimum Inhibitory Concentration (MIC), and Minimum Bactericidal Concentration (MBC) of nanoparticles against *S. mutans***	
	Minimum Inhibitory Concentration (MIC)	**Minimum Bactericidal Concentration (MBC)**
Au NPs	100mg\ml	125mg\ml
Fe NPs	125mg\ml	150mg\ml
Ag NPs	75mg\ml	100mg\ml

According to this existing study, the major processes underlying the antibacterial effects of NPs are may be as follows: disruption of the bacterial cell membrane; generation of ROS; penetration of the bacterial cell membrane; and induction of intracellular antibacterial effects, including interactions with DNA and proteins, appropriate with the study of (Wang,. *et al.*, 2017).

This study focuses on the sensitivity of *S. mutans* to nanoparticles, which indicates the possibility of using this group of nanoparticles as an antibacterial against *S. mutans*. Although the concentrations used in this study differed, they demonstrated positive results in inhibiting bacterial growth and killing it as well.

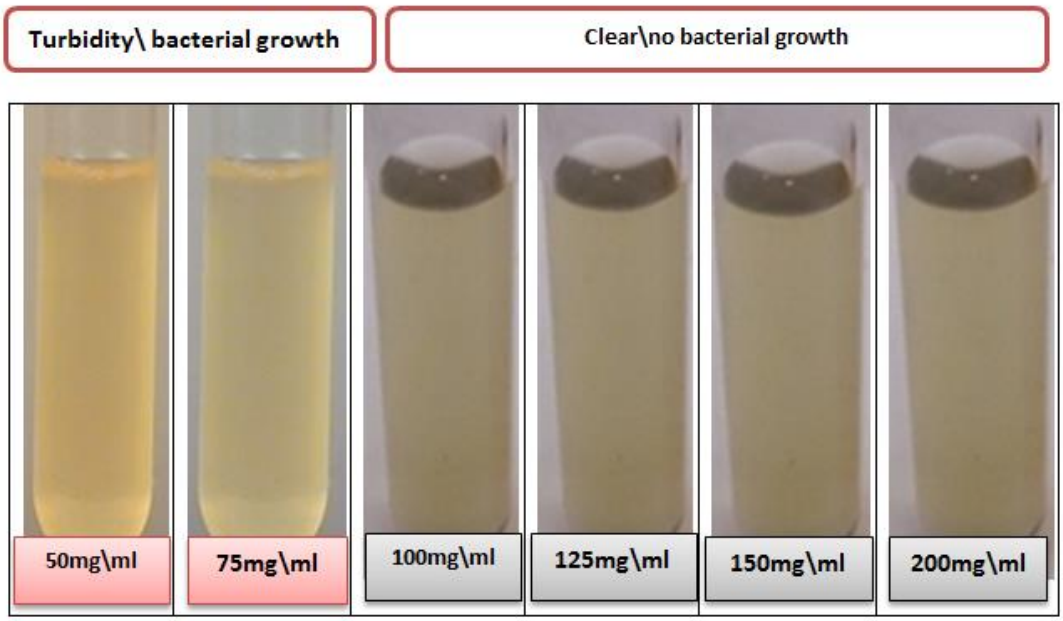


Figure (4.18): MIC of AuNPs against *S. mutans* is 100mg\ml.

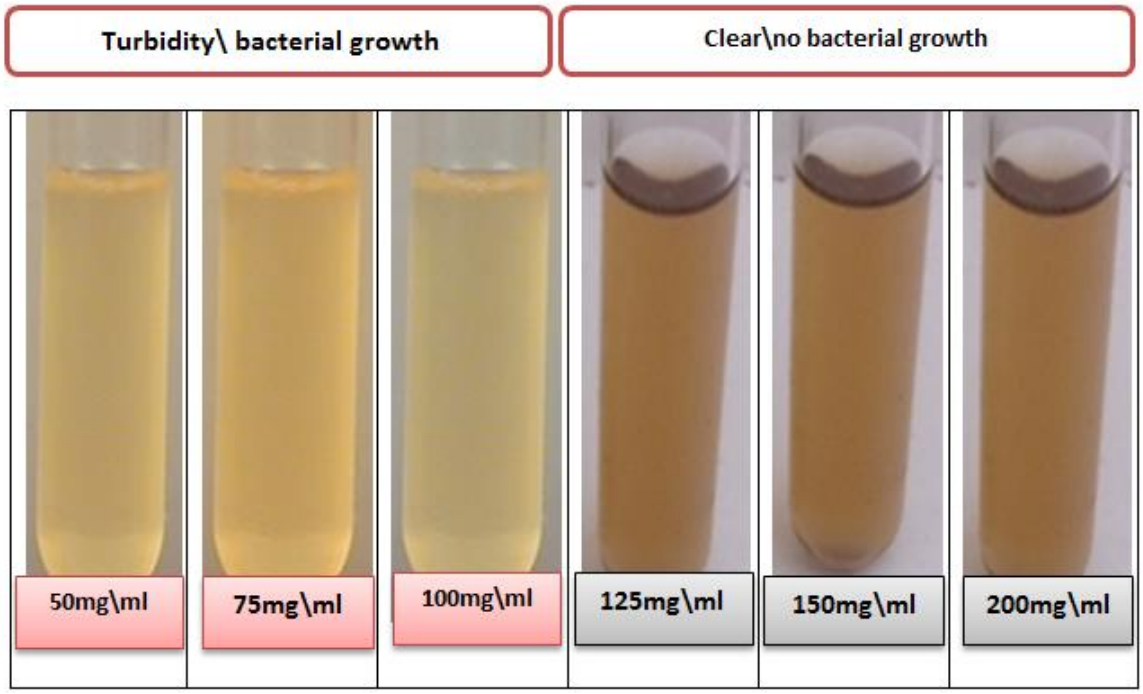


Figure (4.19): MIC of FeNPs against *S. mutans* is 125mg\ml.

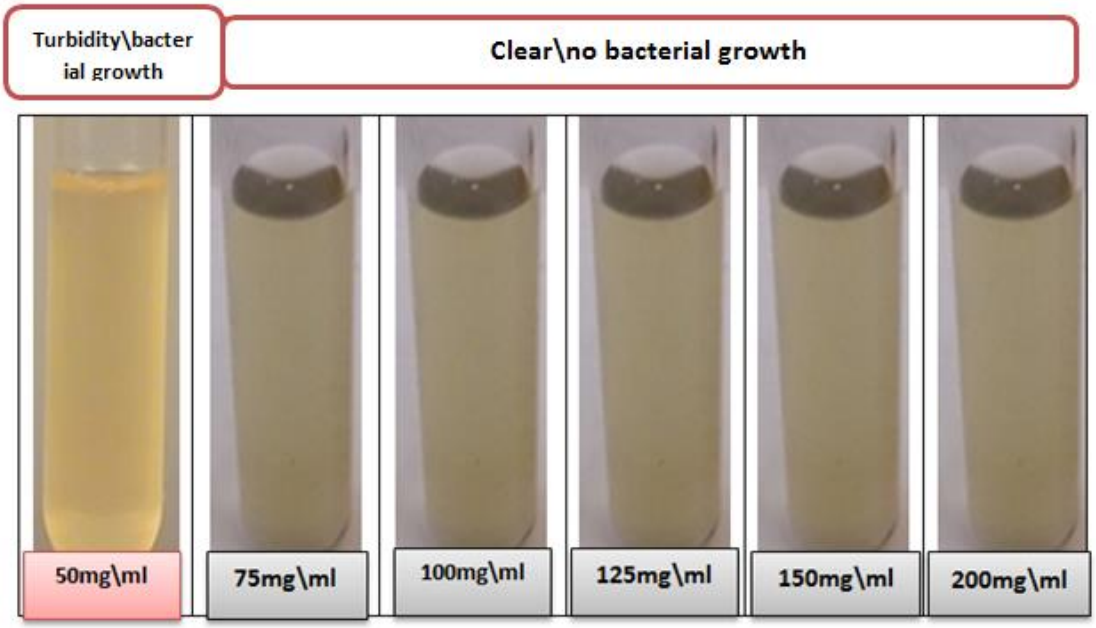


Figure (4.20): MIC of AgNPs against *S. mutans* is 75mg\ml.

This study appropriate to the study of (Li WR,. *et al.*, 2010), which proves that the silver nanoparticles lead to the formation of "pits" in the cell walls of the bacteria and can enter into the periplasm through the pits and destroy the cell membrane. AgNPs not only condense DNA, but may also combine and coagulate with the cytoplasm of damaged bacteria, which results in the leakage of the cytoplasmic component. Silver nanoparticles may cause the condensing of DNA, resulting in a loss of replication and degradation of DNA, thereby inhibiting bacterial growth.

AuNPs have more advantages over chemical or physical methods such as it has significant application in biomedical fields due to its excellent chemical stability, biocompatibility, cost effectiveness, easy preparation, optical properties related with surface plasmon resonance, convenient surface bio-conjugation with molecular probes and low toxicity (E. Boisselier,. *et al.*, 2009). Due to high biocompatibility, chemical stability, convenient surface bio-conjugation with molecular probes, excellent surface plasmon resonance and low toxicity, biosynthesized gold nanoparticles have diverse biomedical applications

including drug delivery, cancer treatment, DNA-RNA analysis, gene therapy, sensing, imaging, and antibacterial agent (Paciotti,. *et al.*, 2009; Groning,. *et al.*, 2001).

Antimicrobial activity of biosynthesized NPs were evaluated against human pathogens gram-negative bacteria, and gram-positive bacteria. These biosynthesized NPs were proved as effective antimicrobial agents against specific human pathogens.

This study agree with the study of (Batool, F. *et al.*, 2021), that said the antimicrobial activity of FeNPs was evaluated by the agar well diffusion method. Zone of inhibitions for FeNPs produced by different concentrations (Same conditions were maintained for each experiment).

But I differ with him in the measurements of zone of inhibition when FeNPs applied, which showed a maximum zone of inhibition of 25 mm against some types of bacteria, and the minimum zone of inhibition is 13 mm. while this study shown maximum zone of inhibition of 20 mm against some types of bacteria and the minimum zone of inhibition is 10 mm. as shown in figure (4.21).

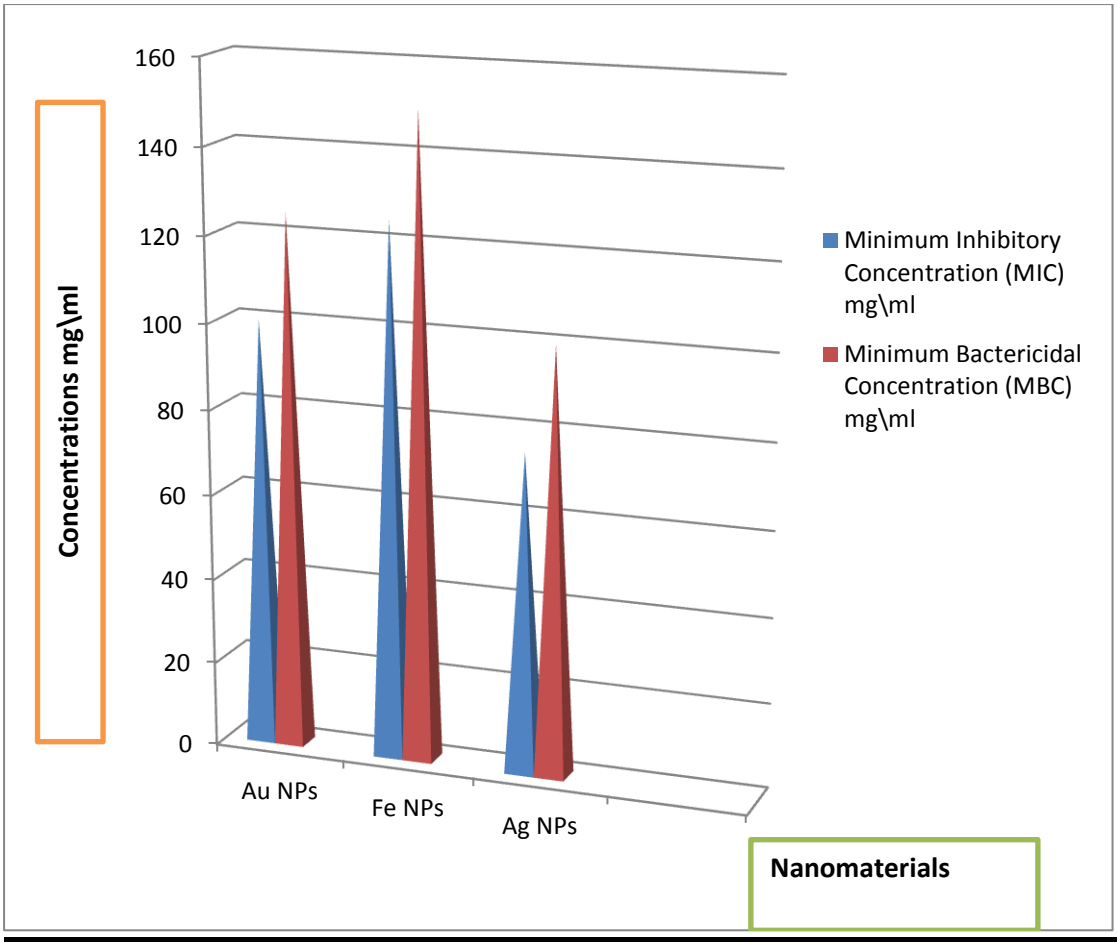


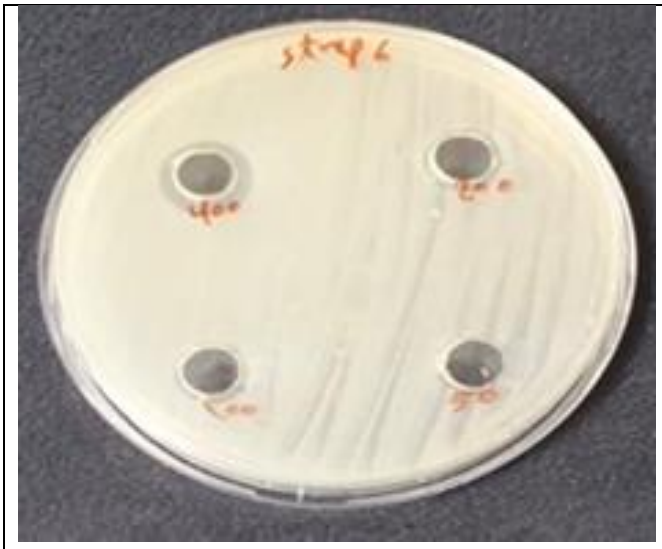
Figure (4.21): Minimum Inhibitory Concentration (MIC), and Minimum Bactericidal Concentration (MBC) of AuNPs, FeNPs, and AgNPs against *S. mutans*.

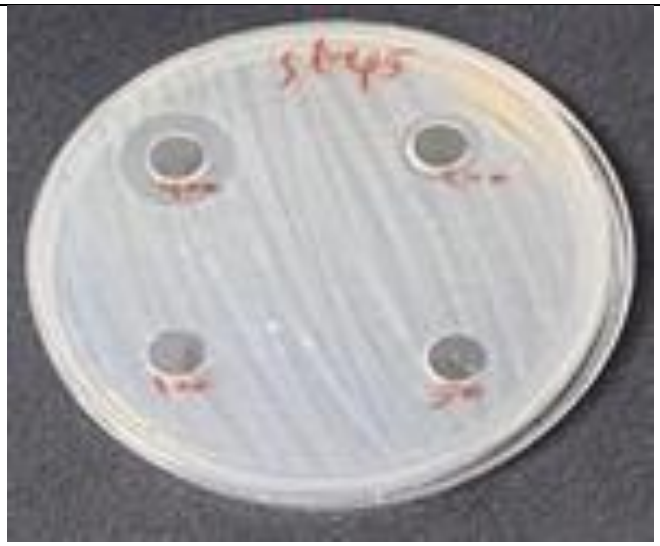
4.8. Anti-bacterial Activity of Nano-materials on *S. mutans*

In this study the inhibition zones of bacterial growth that dipected in each of figure (4.22, 4.23, and 4.24), that the gold, silver and iron nanoparticles were shown a high effective of its antibacterial activity against *S. mutans*. With the application of AuNPs the measurements of inhibition zones ranging between (10 and 21 mm) were recorded. At a concentration of (400 mg/ml) higher than other concentrations, as they range between zone of inhibitions between (18-21mm), followed by the concentration of (200 mg/ml) it was about (16-18mm) inhibition zones. The concentration of (100 mg/ml) was shown about (13-16mm) inhibition zones, while The concentration of (50 mg/ml) was appeared less measurements of inhibition zones, about (10-13mm).

With the application of AgNPs the measurements of inhibition zones ranging between (13 and 20 mm) were recorded. At a concentration of (400 mg/ml) higher than other concentrations, as they range between zone of inhibitions between (17-20mm), followed by the concentration of (200 mg/ml) it was about (16-18mm) inhibition zones. The concentration of (100 mg/ml) was shown about (13-15mm) inhibition zones, while The concentration of (50 mg/ml) was appeared less measurements of inhibition zones, about (13-14mm).

When FeNPs applied against *S. mutans*, the inhibition zone measurements were (16-20mm), (14-17mm), (12-14mm), and (10-13mm) with the concentration of (400 mg/ml) , (200 , (100 mg/ml), and (50 mg/ml) respectively.





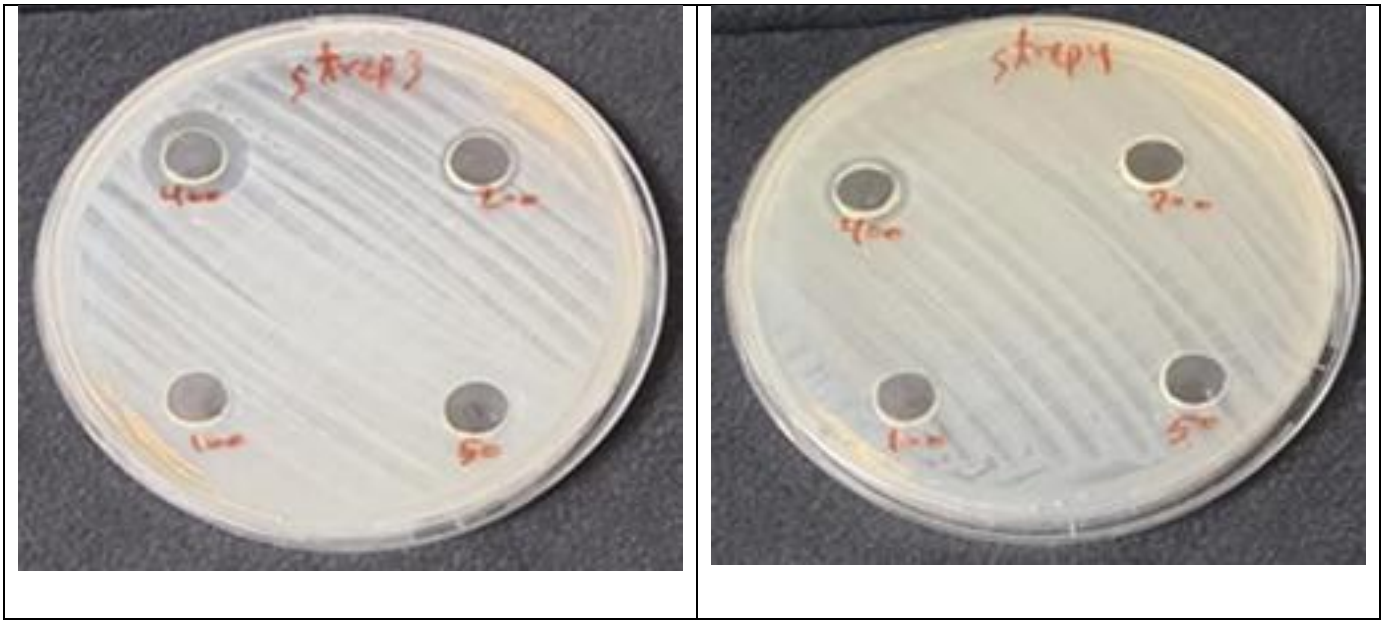
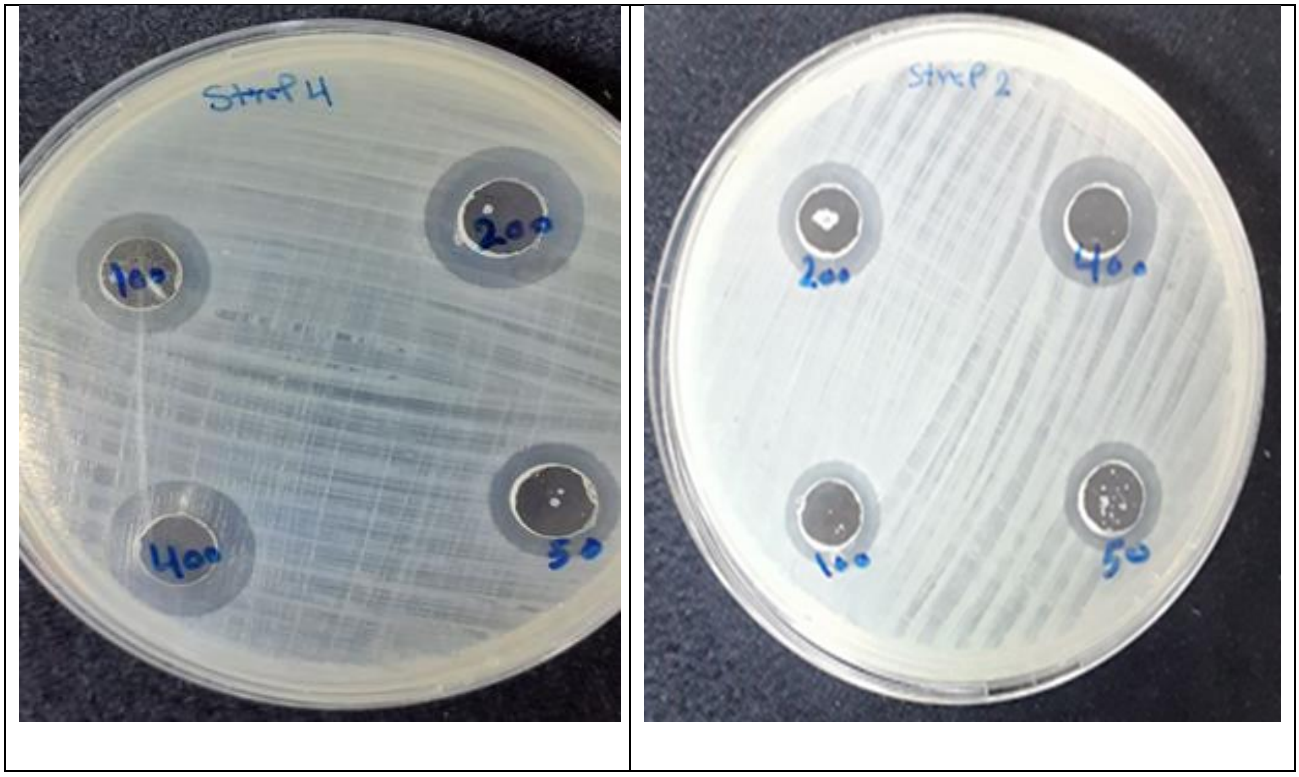


Figure (4.22): The effective of AuNPs against *S. mutans* as antibacterial agent, in (50, 100, 200, 400 mg/ml) concentrations.



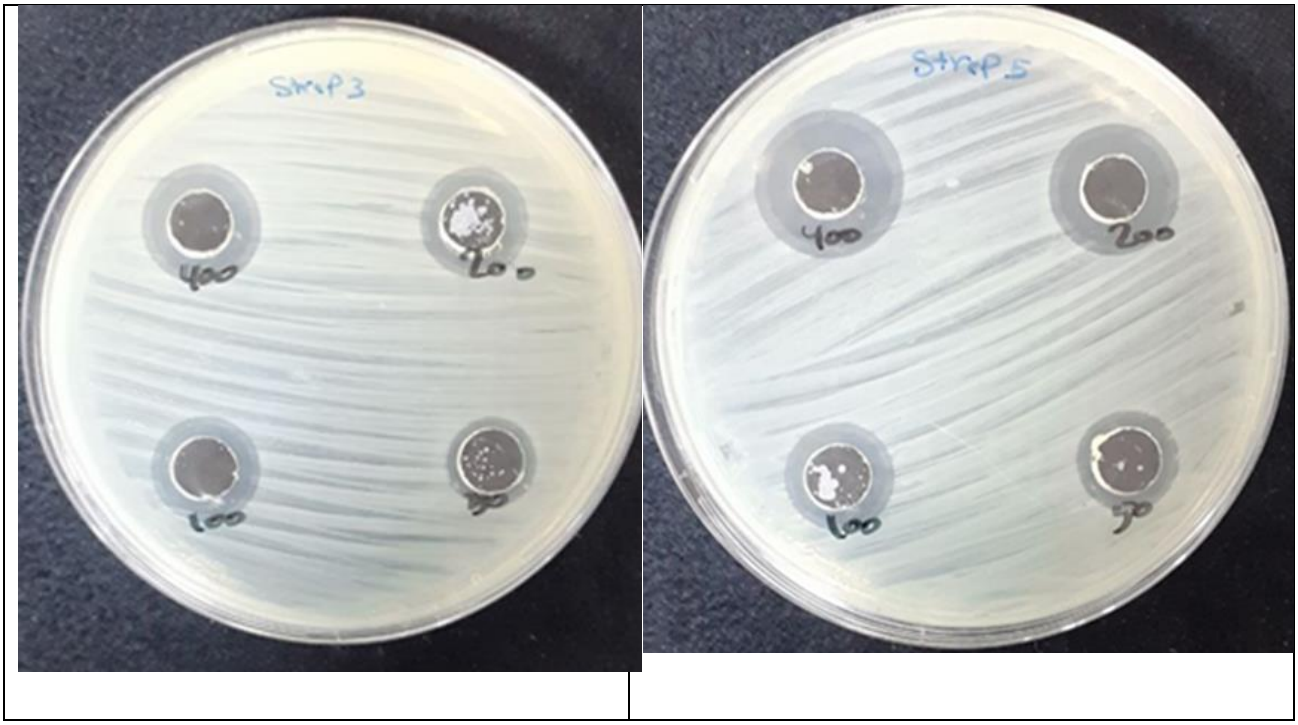
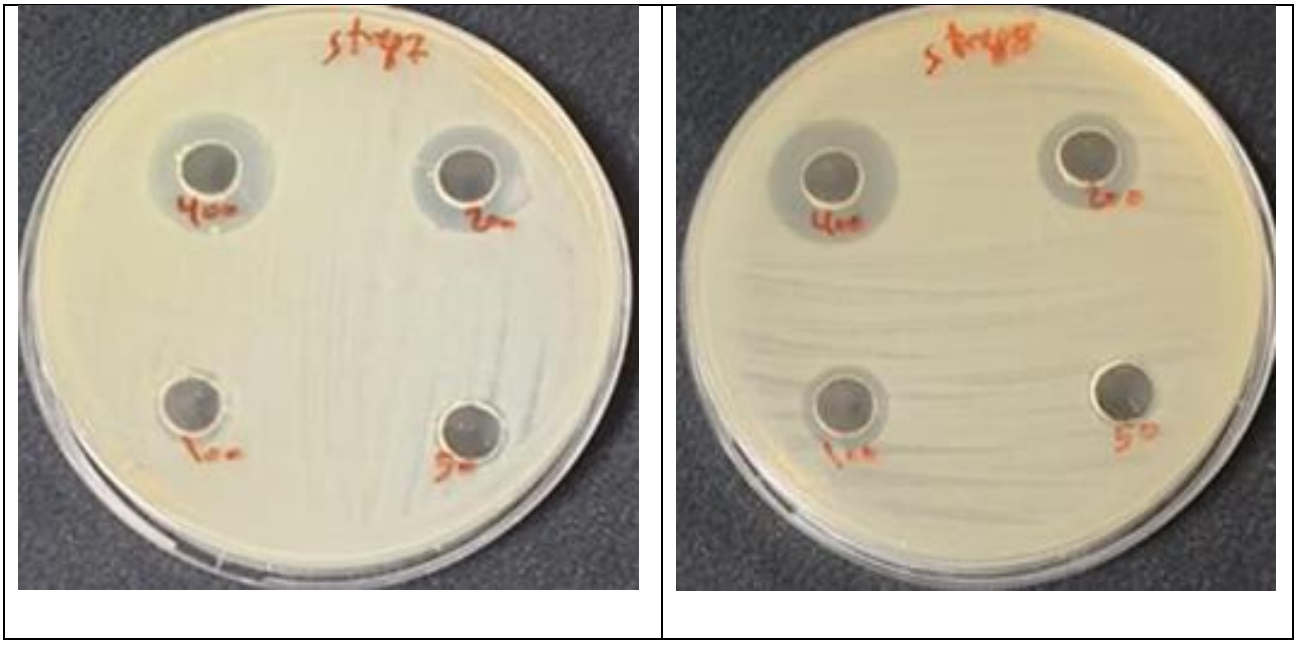
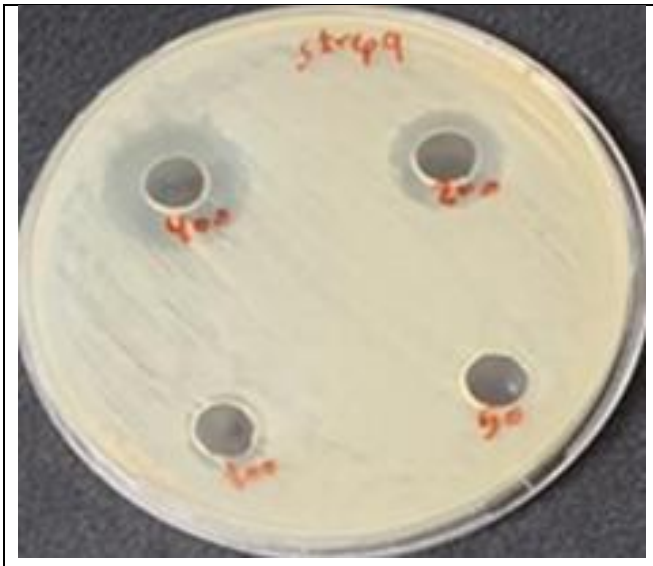


Figure (4.23): The effective of AgNPs against *S. mutans* as antibacterial agent, in (50, 100, 200, 400 mg/ml) concentrations.





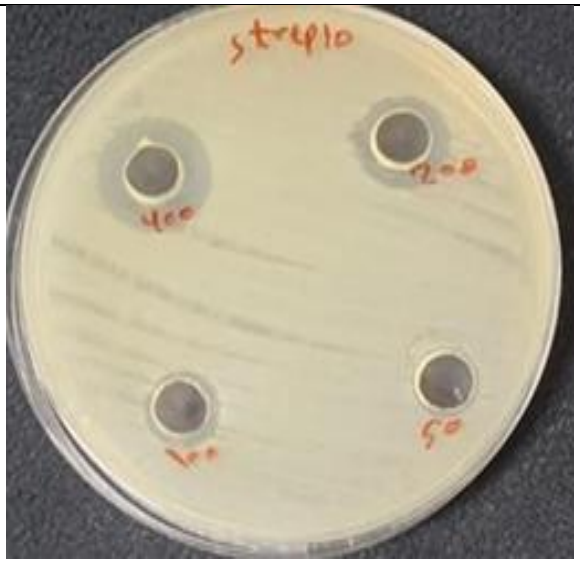


Figure (4.24): The effective of FeNPs against *S. mutans* as antibacterial agent, in (50, 100, 200, 400 mg/ml) concentrations.

However, the detailed antibacterial mechanisms of NPs have not been thoroughly explained, and the same types of NPs often present contrasting effects. The antimicrobial mechanism of action of NPs is generally described as adhering to one of three models: oxidative stress induction (Gurunathan S,. *et al.*, 2012), metal ion release or non-oxidative mechanisms. These three types of mechanisms can occur simultaneously. Certain studies have proposed that Ag NPs prompt neutralization of the surface electric charge of the bacterial membrane and change its penetrability, ultimately leading to bacterial death. Moreover, the generation of reactive oxygen species (ROS) inhibits the antioxidant defense system and causes mechanical damage to the cell membrane.

The AuNPs, FeNPs, and AgNPs used to prevent the growth of *S. mutans* and kill it within the limits of the concentrations that were applied as antibacterial agents showed good results because the these nano-materials possess unique properties due to their high Surface energy,

large proportion of surface atoms, low level of deficiency, and local confinement (Bai et al., 2009).

Conclusions and Recommendations.

Conclusions:

The present study concludes that:

1. Diagnosis the *S. mutans* in dental caries samples, as a pathogenic bacteria through the detection of biofilm formation as a virulence factor.
2. The rate of resistance to multiple antibiotic classes among the *S. mutans* isolates was high in present study.
3. Biosynthesis nanoparticles can be produced nanoparticles economical friendly, strongly effect as antibacterial agents, and showed typical properties and high stability in suspension.
4. Biosynthesis of nanoparticles produces nanoparticles with standard specifications, which perform the required function as an antibacterial and therapeutic agent that kills streptococcal bacteria that cause tooth decay.
5. The AuNPs, FeNPs, and AgNPs have ability to prevent the *S. mutans* growth and kill it..
6. Nanoparticles proved to have a considerable anti-biofilm activity against pathogenic tested bacteria.

Recommendations:

1. Using nanoparticles as anti-bacterial agents against *S. mutans* instead of antibiotics, because bacteria acquire resistance to antibiotics over time.
2. Using biosynthetic nanoparticles as antibacterial agents is commercially better than using antibiotics, due to the ease of their synthesis and the availability of the necessary raw materials for this.

Conclusions and Recommendations

3. The sizes obtained by biosynthesis of nanoparticles are within the range of the required nanoparticle level that performs the desired function of inhibiting and killing *S. mutans*, in addition to the rounded shape of the nanoparticle that is desired.
4. Detecting the roles of gold, silver, and iron nanoparticles in industrial and agricultural applications as well as medical application .
5. The AuNPs, FeNPs, and AgNPs can be used in the form of powder and paste for brushing the teeth, or in the form of mouthwash.

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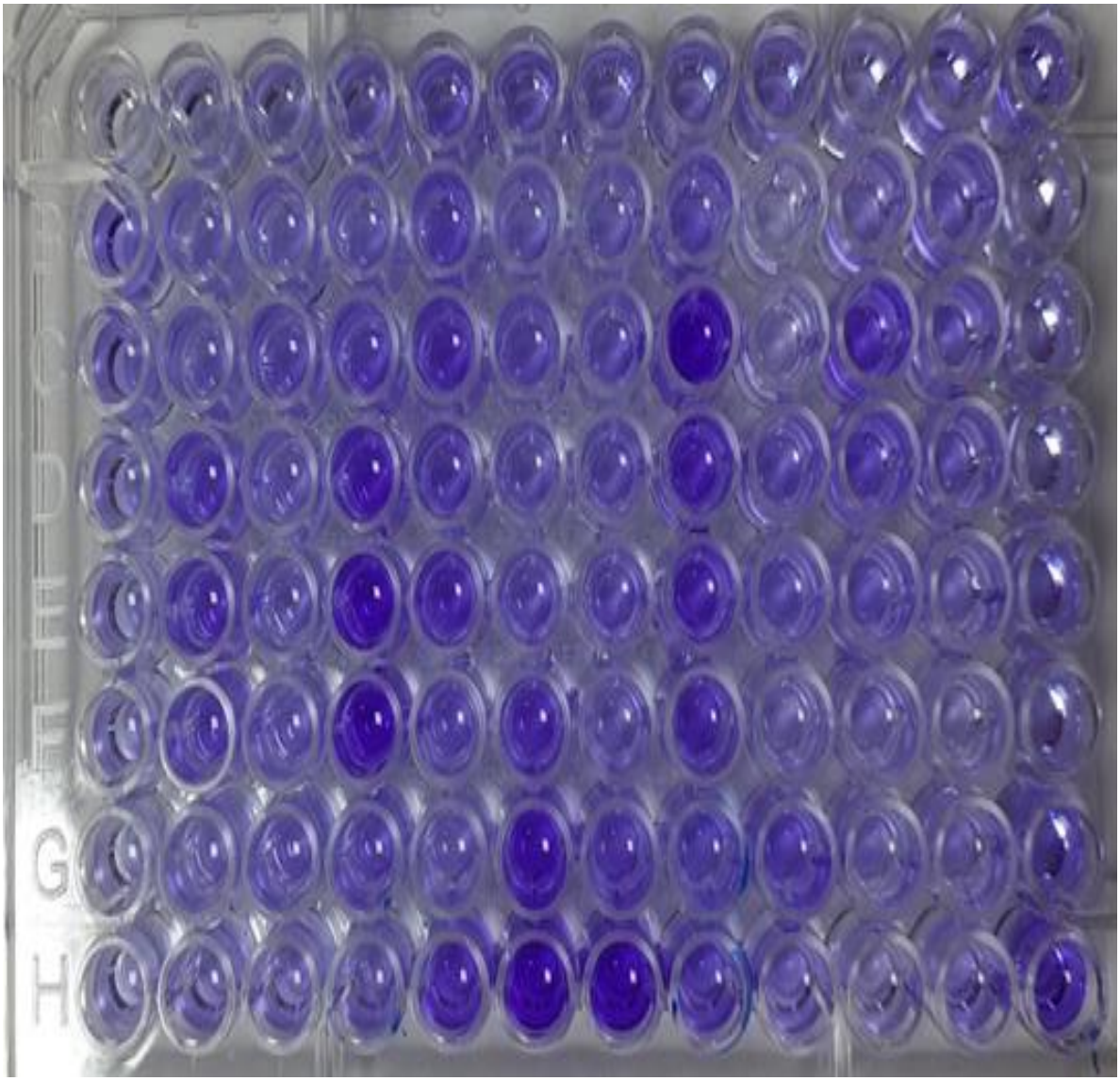
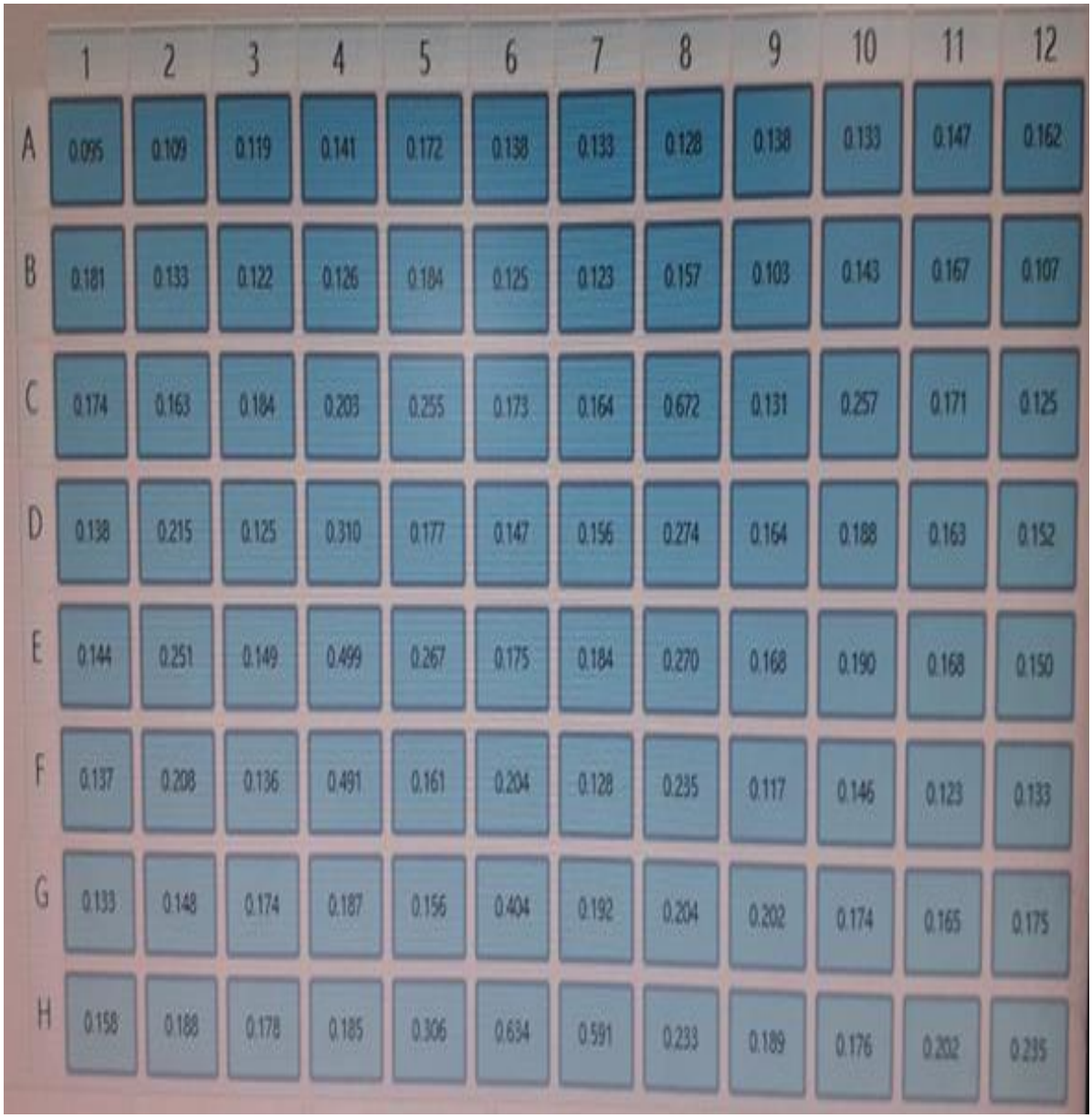
Appendix 1

Biofilm recorded in micro-titer plate:



Appendix 2

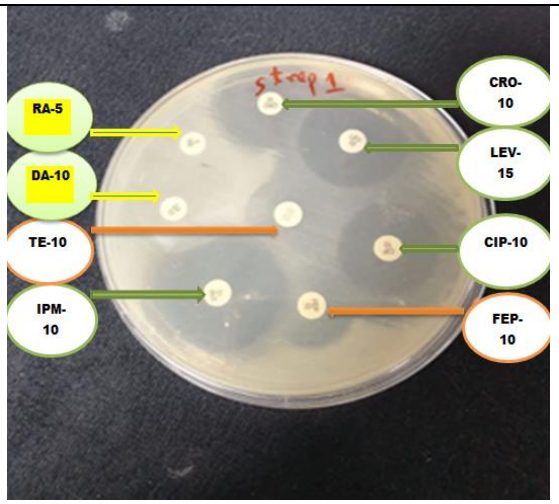
Recorded Sheet of biofilm detection:

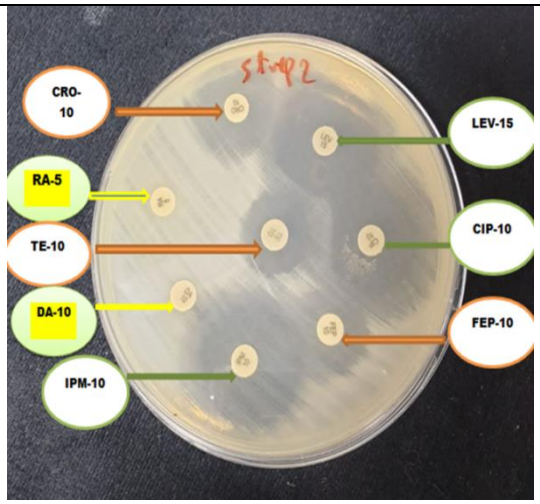


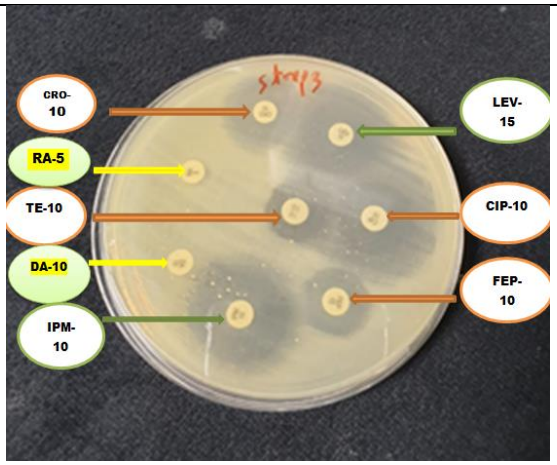
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.095	0.109	0.119	0.141	0.172	0.138	0.133	0.128	0.138	0.133	0.147	0.162
B	0.181	0.133	0.122	0.126	0.184	0.125	0.123	0.157	0.103	0.143	0.167	0.107
C	0.174	0.163	0.184	0.203	0.255	0.173	0.164	0.672	0.131	0.257	0.171	0.125
D	0.138	0.215	0.125	0.310	0.177	0.147	0.156	0.274	0.164	0.188	0.163	0.152
E	0.144	0.251	0.149	0.499	0.267	0.175	0.184	0.270	0.168	0.190	0.168	0.150
F	0.137	0.208	0.136	0.491	0.161	0.204	0.128	0.235	0.117	0.146	0.123	0.133
G	0.133	0.148	0.174	0.187	0.156	0.404	0.192	0.204	0.202	0.174	0.165	0.175
H	0.158	0.188	0.178	0.185	0.306	0.634	0.591	0.233	0.189	0.176	0.202	0.235

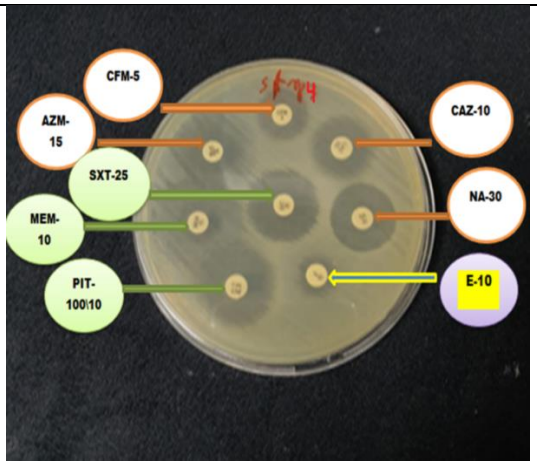
Appendix 3

Antibiotic Susceptibility Tests (AST) to *S. mutans*:



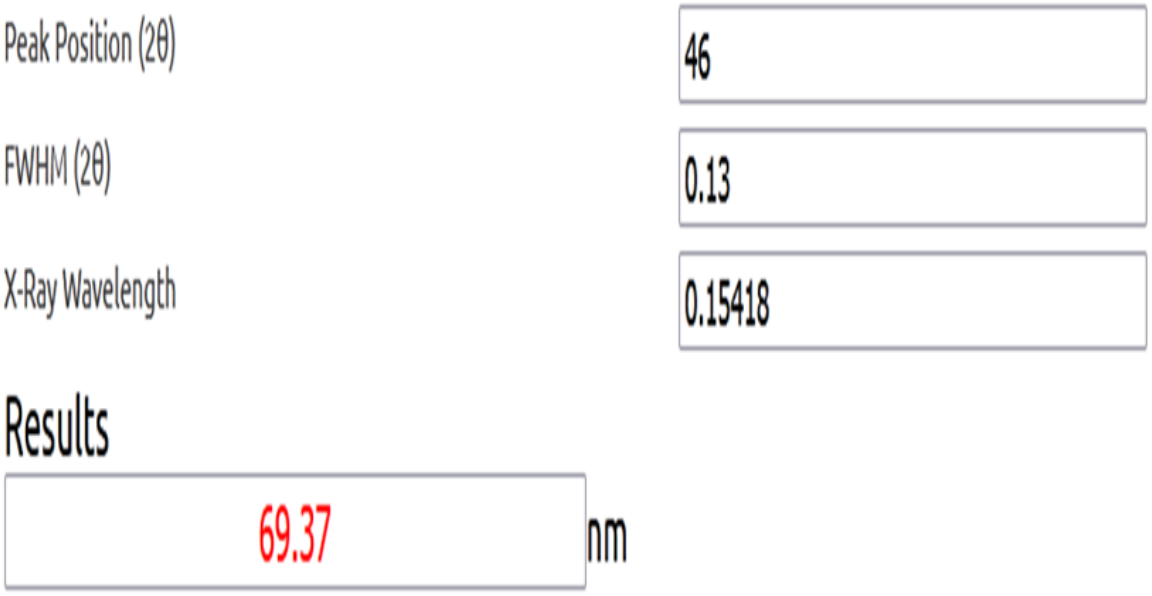






Appendix 4

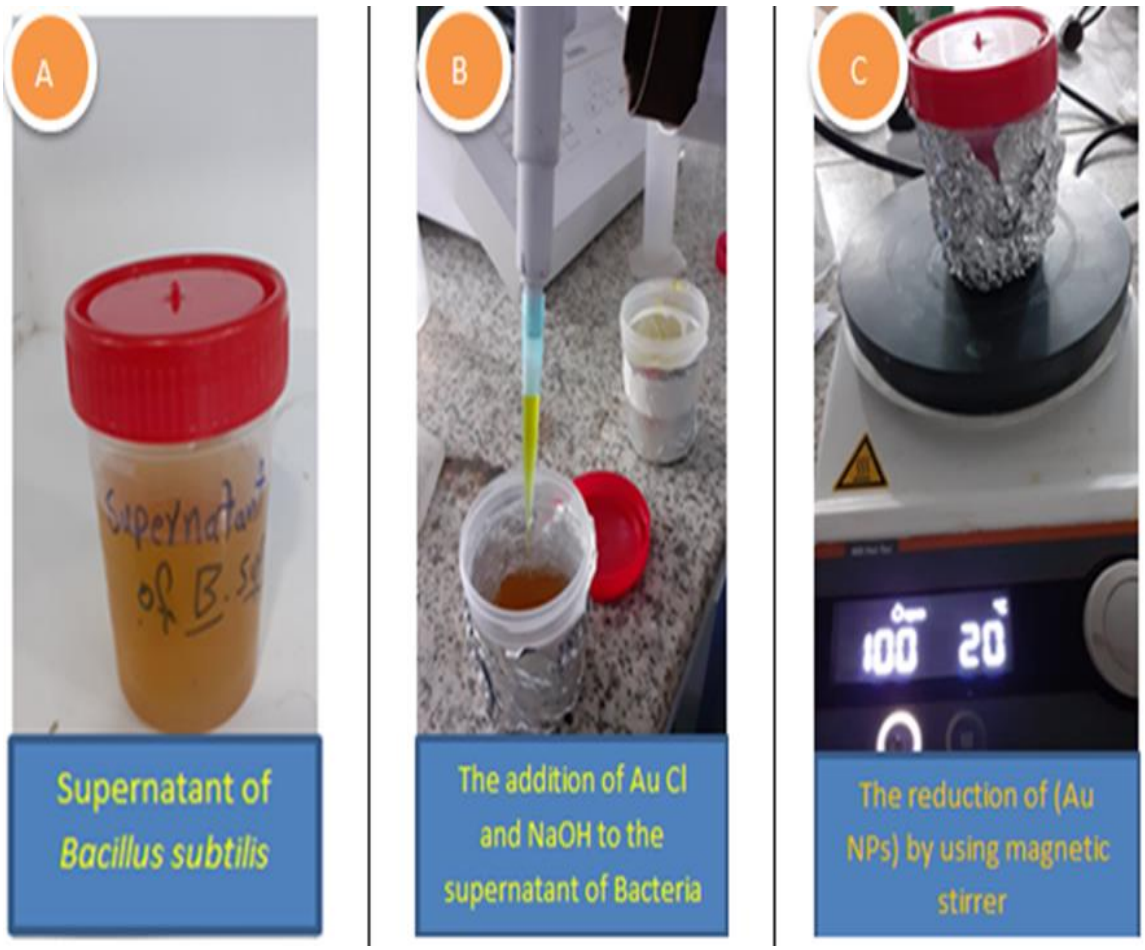
FeNPs size average calculated by (Scherrer Equation):



Appendix 5

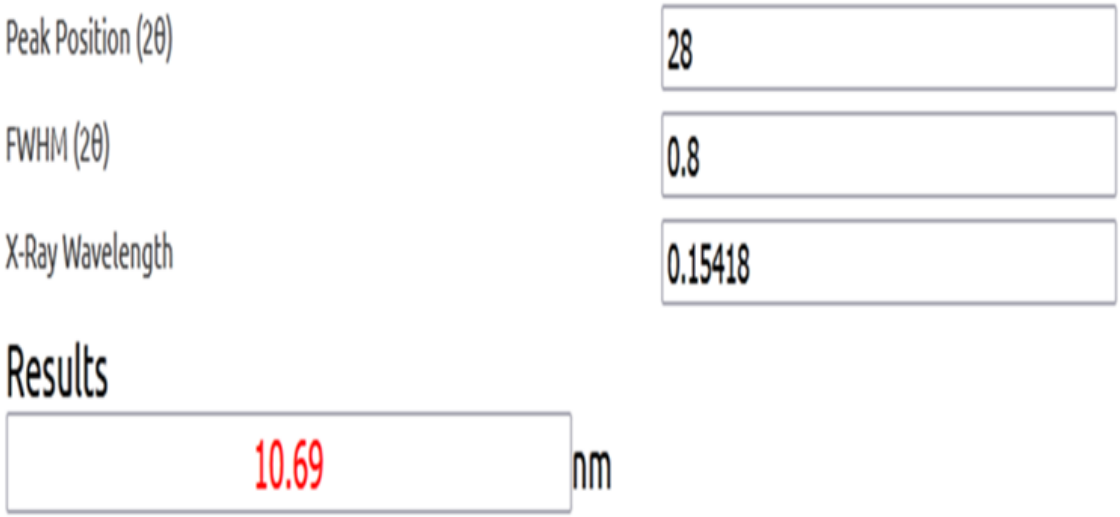
A- the supernatant of *Bacillus subtilis* after centrifugation (with secondary metabolic materials), before the addition of AuCl4. B- the addition of AuCl and NaOH to the supernatant of *Bacillus subtilis* with covering. C- placement of them on the magnetic stirrer for reducing of (AuNPs):

:



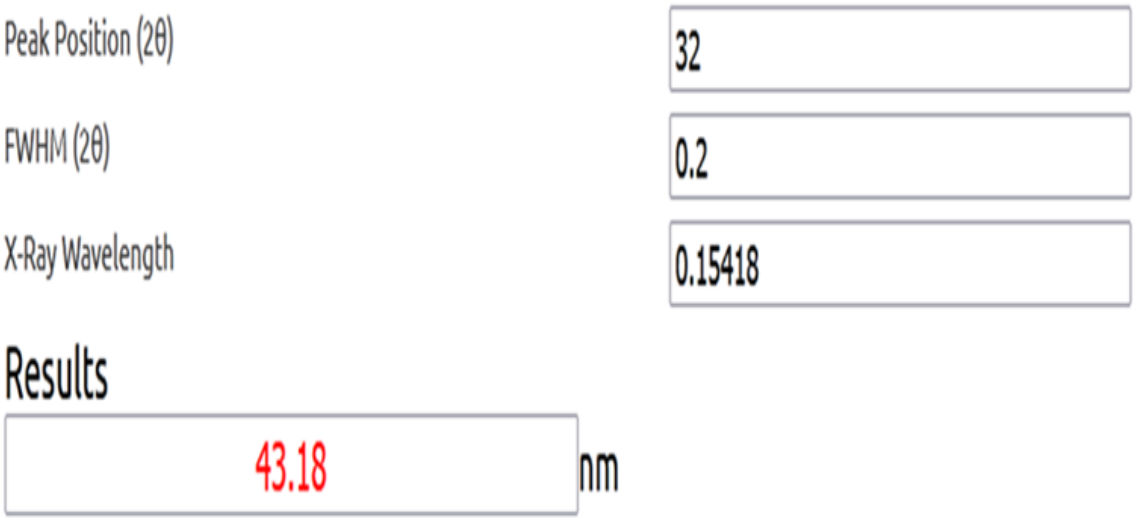
Appendix 6

AuNPs size average calculated by (Scherrer Equation):



Appendix 7

AgNPs size average calculated by (Scherrer Equation):



Appendix 8

The varying sizes of nano-materials (Ag-NPs), (Au-NPs), and (Fe-NPs).

Particle sizes of nano-materials synthesized according to (SEM) measurements (n.m)		
Silver nanoparticles (AgNPs).	Gold nanoparticles (AuNPs).	Iron nanoparticles (FeNPs).
65.00	54.83	70.22
32.93	46.27	67.53
89.69	67.23	84.36
46.36	72.46	73.65
60.53	32.54	34.72
67.26	58.27	52.58
56.37	47.63	82.35
67.70	62.18	46.73

الخلاصة:

بكتريا المكورات العقدية الطافرة *Streptococcus mutans* هي مكورات لاهوائية اختيارية موجبة لصبغة كرام ، و هي بكتيريا مستديرة توجد عادة في تجويف فم الانسان و تساهم مساهمة كبير في تسوس الأسنان. تتضمن الدراسة الحالية الفعالية التضادية لبكتريا المسبحيات العقدية الطافرة للمواد النانوية للذهب و الفضة و الحديد االمصنعة بايلوجياً من البكتريا *Bacillus clausii*، و البكتريا *Bacillus subtilis*، و أصداف المحار على التوالي كعوامل مضادة تمنع نمو و تقتل بكتريا *S. mutans* المعزولة من عينات تسوس الأسنان .

تم فحص خصائص كل نوع من الجسيمات النانوية بواسطة حيود الأشعة السينية XRD ، والمجهر الإلكتروني الماسح SEM ، و تحليل فورييه الطيفي بالأشعة تحت الحمراء FTIR ، للتأكد من شكلها وحجمها ونقاوتها، و كذلك معرفة أن هذه المواد النانوية الخضراء جيدة و تخدم في الغرض المطلوب . و كانت نتيجة معادلة شيرير المطبقة لقياسات حجم الجسيمات للفضة Ag-NPs و الذهب Au-NPs و الحديد Fe-NPs تساوي: 10.69، 43.18، و69.37 نانومتر، على التوالي.

تم جمع مئة عينة من تسوس الأسنان بواسطة الوسط الناقل للبكتريا من المرضى الذين قاموا بزيارة العيادات التعليمية لكلية طب الأسنان / جامعة بابل خلال الفترة من نيسان \ 2022 الى آب \ 2022 ، و تم زرع هذه العينات على الوسط الاختياري Mitis Salivarius Agar فتبين أن 64 عينة لم تُظهر نمو بكتيرياً ، بينما اظهرت 36 عينة نمواً بكتيرياً واضحاً تمثل بظهور مستعمرات بكتيرية واضحة. بعد ذلك تم عزل و تشخيص البكتريا باستخدام طرق مختلفة تمثلت في استخدام صبغة كرام متبوعة بالفحص المورفولوجي باستخدام المجهر الضوئي ، و تم تأكيد تحديد هوية *S. mutans* من خلال تقنية فحص تفاعل البلمرة المتسلسل PCR عن طريق تضخيم الجيني المحدد لتسلسل الجينات للجين *Sm479* الخاص بالبكتريا *S. mutans*.

تم الكشف عن الأغشية الحيوية باستخدام تقنية مقايسة الممتز المناعي المرتبط بالإنزيم ELISAبواسطة لوحة microtitter ، وشملت النتيجة 14 عزلة غير ملتصقة، 61 عزلة ضعيفة التصاق، 16 عزلة متوسطة التصاق، و 4 عزلة قوية التصاق)، حوالي 14.73%، 64.21% ، 16.84%، و4.21% على التوالي.

حساسية و مقاومة بكتيريا *S. mutans* لـ 17 صنف من المضادات الحيوية اجريت من خلال "اختبار الحساسية للمضادات الحيوية "(AST) على 36 عزلة ، حيث أظهرت النتائج ان نسب

مقاومة البكتريا للمضادات تراوحت بين 22.22%-100%، و معتدلة بنسب تراوحت بين (لا يوجد -36.11%)، في حين بلغت النسب الحساسة (لا يوجد -41.66 %).

تم إجراء النشاط المضاد للبكتيري للمواد النانوية على البكتريا ، ضد إنتاج الأغشية الحيوية المضادة واختبار انتشار قرص الأجار، وسجلت جميع الجزيئات النانوية فعالية عالية كمضادات بكتيري تعمل على تثبيط نمو *S. mutans* ، فسجلت جزيئات الذهب النانوية بتركيز 400 ملغم/مل أعلى من التراكيز الأخرى، حيث تراوح بين مناطق التثبيط بين 18-21 ملم، يليه تركيز 200 ملغم/مل فكانت حوالي 16-18 ملم مناطق تثبيط، في حين أظهرت بتركيز 100 ملغم/مل مناطق تثبيط 13-16 ملم، بينما اظهرت في التركيز 50 ملغم/مل أقل قياسات لمناطق التثبيط بلغت حوالي 10-13 ملم. عند تطبيق جزيئات الفضة النانوية تم تسجيل قياسات مناطق التثبيط التي تتراوح بين 13 و 20 ملم ، و بتراكيز 400 ملغم/مل أعلى من التراكيز الأخرى، حيث بلغت مناطق التثبيط حوالي 17-20 ملم، يليه تركيز 200 ملغم/مل فكانت حوالي 16-18 ملم مناطق تثبيط .أظهر التركيز 100 ملغم/مل مناطق تثبيط 13-15 ملم، في حين اظهر تركيز 50 ملغم/مل أقل في قياسات مناطق التثبيط حوالي 13-14 ملم .عندما تم تطبيق جزيئات الحديد النانوية كانت قياسات منطقة التثبيط هي 16-20 ملم، 14-17 ملم، 12-14 ملم، 10-13 ملم بتركيزات 400 ملغم/مل، و 200، و 100 ملغم/مل و 50 ملغم/مل على التوالي .

تم اجراء اختبارات التركيز المثبط الأدنى (MIC) لـ AuNPs و FeNPsو AgNPs ضد البكتيريا العقدية .تم إجراء وتسجيل الطفرات 100، 125، و 75 ملغم/مل، على التوالي .في حين أن الحد الأدنى لتركيز مبيد الجراثيم (MBC) من AuNPs و FeNPsو AgNPs ضد البكتيريا العقدية .الطافرة 125، 150، و100 ملغم/مل على التوالي.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة بابل كلية العلوم
قسم علوم الحياة

الفعالية التضادية لبكتريا المسبحيات العقدية للمواد النانوية للفضة و الذهب و الحديد المصنعة بايلوجياً

اطروحة مقدمة الى

مجلس كلية العلوم / جامعة بابل وهي جزء من متطلبات نيل شهاة الدكتوراه

فلسفة في العلوم / علوم الحياة

من قبل

حسنين جواد عبدالحسين حسين

بكالوريوس علوم حياة عامة /جامعة بابل/ 2000م
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بأشراف

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2023 م

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