Plant Archives Vol. 21, Supplement 1, 2021 pp. 579-585 e-ISSN:2581-6063 (online), ISSN:0972-5210

Plant Archives

Journal homepage: http://www.plantarchives.org
doi link : https://doi.org/10.51470/PLANTARCHIVES.2021.v21.S1.088

PRODUCTION AND CHARACTERIZATION OF SILVER NANOPARTICLES BY *LACTOBACILLUS* SPP. AND DETERMINE INHIBITORY EFFECT AGAINST GROWTH OF PATHOGENIC BACTERIA

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ABSTRACT

The present study aimed to produce silver nanoparticles by the Biosynthesis method which was a very important area due to their economic and ecofriendly benefits. The current research was used the whey of local isolates of *Lactobacillus spp*. were isolated and identified from different types of local dairy products in Baquba city markets. Silver nanoparticles were Characterized using X-ray diffraction (XRD) analysis confirms the nanoparticles are silver and silver chloride (AgCl) cubic types and by using the(crystalline) domains equation, three strongest peaks, the calculated particle size were 17.1and 18.6 nm for Ag, and 24.3 nm for and AgCl. Atomic Force Microscopy (AFM) is used for size, topography, and granularity volume distribution of biosynthesized nanoparticles Ag/Agcl average size 50 nm. UV-visible spectroscopy revealed the formation of AgNPs. Fourier transform infrared (FTIR) spectroscopy shows different functional groups of biomoleculesare responsible for the reduction and capping process. Electron microscopic (SEM) was used to characterize the shape and size of the nanoparticles.

Determination of the antibacterial activity of silver and silver chloride nanoparticles against the selected multiple drugs resist (MDR) bacteria by agar well diffusion method. It was observed that the growth of these bacteria was inhibited from 12.5 mg/ml Ag & Agcl NPs concentration and the nanoparticles concentrations (12.5,25,50,100 mg/ml) had a maximum inhibitory effect against *S. aureus* (24, 28, 30, 32 mm respectively) and minimum inhibitory effect against *E. coli* (16, 21, 23, 27 mm respectively) while the others bacteria (*P. mirabilis, S. epidermidis, K. pneumoniae, and P. aeruginosa*) inhibitory effect varied between the bacterial isolates and the concentration of the silver nanoparticles.

***Keywords*:** Sliver nanoparticles, Lactobacillus,XRD, FT-IR, pathogenic bacteria

Introduction

Infectious diseases remain one of the leading causes of morbidity and mortality worldwide. The WHO and CDC have expressed serious concern regarding the continued increase in the development of multidrug resistance among bacteria. Therefore, the antibiotic resistance emergency is one of the most demanding issues in global public health. Associated with the rise in antibiotic resistance is the lack of new antimicrobials. This has prompted creativities worldwide to develop novel and more effective antimicrobial compounds as well as to develop novel delivery and targeting strategies. Nanoparticles display unique physical, chemical and biological properties in term of their size, reactivity, surface area to volume ratio, magnetic and optical properties. The reduction of materials' dimension has pronounced effects on the physical properties that may be significantly different from the corresponding bulk material (Ahmad, 2003). Physical and chemical methods for nanoparticle synthesis are expensive and involve the production of toxic by-products which are environmentally not safe methods. An alternative method for synthesizing nanoparticles depends on the biological system and the use of microbes as a tool for the synthesis of new functional nanomaterials has gained much interest in recent times (Phanjom and Ahmed, 2015). Biosynthesis of nanoparticles is a kind of bottom-up approach where the main reaction occurring is reduction/oxidation (Silambarasan and Abraham, 2012). In recent times, a simple and viable substitute to more complex chemical synthetic procedures to get nanomaterial's known as a biosynthetic method employing both biological microorganisms such as bacteria (Joerger *et al*., 2000) and fungus (Shankar *et al.,* 2003) or plants extract (Huang *et al*., 2007; Gardea-Torresdey *et al*., 2002; Chandran*et al*., 2006) have used. Culture supernatant of microorganisms may act both as reducing and capping agents in Silver NPs production. The cell-free extracts have been found to contain environmentally compassionate, yet chemically complex biomolecules such as enzymes or proteins, amino acids, polysaccharides, and vitamins which may be responsible for the reduction of Ag+ ions (Collera-Zuniga *et al*., 2005). Silver and its compounds are known to have antimicrobial properties. Early in the 19th century, 0.5% $AgNO_3$ was used for the treatment and prevention of microbial infections (Hwang *et al*., 2012). It is widely believed that silver nanoparticles are incorporated in the cell membrane, which causes leakage of intracellular substances and eventually causes cell death. Some of the silver nanoparticles also penetrate into the cells. It is reported that the bactericidal effect of silver nanoparticles decreases as the size increases and is also affected by the shape of the particles (Dhanalakshmi *et al*., 2012). The bacteria that produce lactic acid as their major or sole fermentation product. They belong to the low G+C Gram-positive bacteria, Class Bacilli and Order Lactobacillales. They are fastidious nutritionally. Energy is obtained by these bacteria through substrate-level phosphorylation. They are facultative anaerobes and are

sometimes classified as aerotolerant anaerobes (Willey *et al*., 2008). They are one of the important groups of microorganisms in food fermentation and they produce a variety of antimicrobial compounds such as ethanol, formic acid, acetone, hydrogen peroxide, diacetyl, and bacteriocins which conferpreservative ability on them as a natural competitive means to overcome other microorganisms sharing the same niche (Oliveira *et al*., 2008). This study is aimed to the production, characterization and antibacterial prospective of silver nanoparticles produced from culture-free extracts of lactobacillus spp. bacteria.

Materials and Methods

Sources of Pathogenic Bacteria Isolates

Pathogenic bacteria are isolated from different clinical sources(burn, wounds, and UTI). A total of (200) samples were cultured on selective and differential media.The bacteria identified according to morphological characters (as primary diagnostic tests) of isolates grown on MacConkey agar and Blood agar, as well as identified by biochemical tests including Oxidase, Catalase, IMiVC, Kliger's iron, and Urease production tests. Finally,confirm the identification of bacterial isolate by VITEK-2 system.

Antimicrobial susceptibility test

All the bacterial isolates were tested for antimicrobial susceptibility according to the CLSI (2019), criteria by using disc diffusion method.

Isolation of *Lactobacillus spp.*

Lactobacillus spp. bacteria isolated from local fermented and curd food (dairy products) collected from various local markets in Baaquba city, and isolated by serial dilution technique using MRS Broth and Agar(Ranganath*et al*., 2012). The bacterium was identified based on cultural and biochemical characteristics. Initially all of the isolates were examined for Gram staining and catalase production. Then cell morphology and colony characteristics on deManRogosa Sharpe agar (MRS agar) were tested and the isolates were separated into different phenotypic groups.

Screening of *Lactobacillus spp.* for silver nanoparticle biosynthesis

Lactobacillus spp. isolates were inoculated into sterilized 250 ml of home delivered milk (fat free) in 500 ml Erlenmeyer flask for curdling at 37°C for 24 hours. The whey was collected by coarse filtration (Whatman0.4).The filtrate was pale yellow in appearance, and the pH was typically 4.4.Five mL of each filtrate whey solution taken in a test tube, 5 mg of $AgNo_3$ was added and kept in the laboratory under ambient conditions (Nair and Pradeep, 2002). The solution became brown in about 12 h. A brown mass gets deposited at the bottom of the test tube after 24 h., control was run along with experimental tubes.After the precipitate was formed, each tube containing the precipitate was centrifuged to ensure sedimentation of all the nanoparticles.Centrifugation was done at 8000rpm for 5 minutes. In order to obtain a pure powder first, the product was dried at 50 °C for 24 hours, and then was calcination at 150° C for 3 hours. The calcination process was carried out according to Goudarzi *et al*. (2016).

Characterization of Silver Nanoparticles

The characterization of Ag NPs was carried out by using X-ray diffractometer (XRD) pattern analysis. The molecular analysis of the samples was performed by Fourier Transform Infra-Red Spectroscopy (FT-IR) and UV-Visible Spectrophotometer. The morphology analysis and particle size of samples were carried out by Emission Scanning Electron Microscope (SEM). The size, topography and granularity volume distribution of biosynthesized nanoparticles are characterized by the use Atomic Force Microscopy (AA-3000, Shimadzu Japan).

Determination the antibacterial activity of Silver nanoparticles in *vitro*

Determination of Silver nanoparticles antibacterial activity was done by agar wells diffusion assay according to Obedat *et al*. (2012). Antibacterial activity was determined against multiple drugs resist (MDR) bacteria. First a stock of Ag NPs in concentration 100% was prepared by dissolved 280 mg of NPs powder in 2.8 ml of deionized water (the dissolve process was done by using vortex and heating the solution in water bath (40 °C to make sure that all powder was dissolved completely).Four different concentrations were prepared from the stock (12.5,25,50,100) mg/ml. Each bacterium was cultured on Muller Hinton agar after comparison with McFarland tube ($0.5x10^8$) CFU/ml by streaking method and the five wells 5mm were made in the plate by sterilizedcork borer, four different concentrations of silver nanoparticles 100 µl were added to wells, the fifth well was take as control by add 100 µl of deionized water. Three repeats made of for each bacterium and then incubated at 37 °C for 24 hours. The efficacy of each concentration of nanoparticles was determined by measuring the inhibiting diameter of each concentration by a standard ruler in millimeters.

Results and Discussion

Isolation and identification of bacteria

Bacterialgenus and species identified after the initialdiagnosis, from the total two hundredcultured swabs, the results appeared; *Klebsiella pneumoniae*, *Escherichia coli*, *Proteus mirabilis, Pseudomonas aeruginosa, Staphylococcus aureus* and *Staphylococcus epidermidis* as shown in table (1 and 2).

Table 1: Identification of Gram-Negative Bacteria by Biochemical tests

Biochemical Tests	*Proteus mirabilis*	*Pseudomonas aeruginosa*	*Escherichia coli*	*Klebsiella pneumoniae*
Catalase	(+ve)	(+ve)	(+ve)	(+ve)
Oxidase	(-ve)	(+ve)	(-ve)	(-ve)
Indole	(-ve)	(-ve)	(+ve)	(-ve)
Methyl red	(+ve)	(-ve)	(+ve)	(-ve)
Vogas-Proskauer	(-ve)	(-ve)	(-ve)	(+ve)
Citrate utilization	(+ve)	(+ve)	(-ve)	(+ve)
Kliger's iron	K/A H_2S produce	K/K --	A/A ++	A/A ++
Urease production	(+ve)	(-ve)	(-ve)	(+ve)

Table 2: Identification of Gram positive Bacteria *Staphylococcus spp.* by Biochemical tests.

Biochemical tests	*S. aureus*	*S. epidermidis*
Mannitol salt agar	Yellow colonies	Pink colonies
Coagulase test	(+)	(-)
Oxidase test	(-)	(-)
Catalase test	(+)	(+)

Antimicrobial susceptibility test

All the bacterial isolates were tested for antimicrobial susceptibility according to the CLSI (2019), criteria by using agar diffusion method. The multi drug resistance (MDR) bacteria were selected forfurther study, those bacteria were: *E. coli* which appeared resistance to Imipenem, Aztreonam, Cefpodoxime, Trimethoprim- sulfamethoxazole, Azithromycin, Ampicillin-sulbactam, Ticarcillin-clavulanate, Cefuroxime, Cefoxitin and sensitive for Tetracycline, Doxycycline, Levofloxacin, *K. pneumonia* showed resistanceto Ciprofloxacin, Ofloxacin, Gentamicin, Trimethoprim, Cefotaxime, Cefuroxime, Amikacin, Streptomycin, Nitrofurantoin, Piperacillin, Imipenem. *P. mirabilis*resist to Gentamicin, Trimethoprim, Cefotaxime, Cefuroxime, Amikacin, Streptomycin, Nitrofurantoin, Chloramphenicol and sensitive to Ciprofloxacin, Ofloxacin, Piperacillin, Imipenem. *P. aeruginosa*resist to Gentamicin, Tobramycin, Aztreonam, Amikacin, Ceftazidime, Piperacillin, Imipenem, Cefepimeand sensitive for Ciprofloxacin, Ofloxacin and Levofloxacin.

S. aureus resist for Ciprofloxacin, Ofloxacin, Levofloxacin, Azithromycin, Clarithromycin, Tetracycline, Streptomycin, Gentamicin, Vancomycinand sensitive to Doxycycline, Clindamycin, Chloramphenicol, *S. epidermidis* resist to Ciprofloxacin, Ofloxacin, Levofloxacin, Azithromycin, Clarithromycin, Tetracycline, Doxycycline, Gentamicin,Vancomycin and sensitive to Clindamycin, Streptomycin, Chloramphenicol as shown in table 3 and 4.

Table 3: Antimicrobial susceptibility test for Gram-Negative isolates (MDR)

Antibiotics	*E. coli*	Antibiotics	*K. pneumonia*	*P. mirabilis*
Imipenem	R	Ciprofloxacin	R	S
Tetracycline	S	Ofloxacin	R	S
Doxycycline	S	Gentamicin	R	R
Aztreonam	R	Trimethoprim	R	R
Levofloxacin	S	Cefotaxime	R	R
Cefpodoxime	R	Cefuroxime	R	R
Trimethoprim- sulfamethoxazole	R	Amikacin	R	R
Azithromycin	R	Streptomycin	R	R
Ampicillin-sulbactam	R	Nitrofurantoin	R	R
Ticarcillin-clavulanate	R	Piperacillin	R	S
Cefuroxime	R	Imipenem	R	S
Cefoxitin	R	Chloramphenicol	R	R

Table 4: Antimicrobial susceptibility test for MDR Gram positive (*S. aureus* and *S. epidermidis*) and *pseudomonas aeroginosa*

Antibiotics	*P. aeruginosa*	Antibiotics	*S. aureus*	*S. epidermidis*
Ciprofloxacin	S	Ciprofloxacin	R	R
Ofloxacin	S	Ofloxacin	R	R
Gentamicin	R	Levofloxacin	R	R
Tobramycin	R	Azithromycin	R	R
Aztreonam	R	Clarithromycin	R	R
Levofloxacin	S	Tetracycline	R	R
Amikacin	R	Doxycycline	S	R
Meropenem	R	Clindamycin	S	S
Ceftazidime	R	Streptomycin	R	S
Piperacillin	R	Gentamicin	R	R
Imipenem	R	Vancomycin	R	R
Cefepime	R	Chloramphenicol	S	S

Biosynthesis of silver nanoparticles by *Lactobacillus spp.*

*Lactobacillus spp.*were screened for production of (AgNPs) using their extracellular (cell free supernatant) of bacterial cultures in skim milk (PH 5 and 37 C). The primary sight for AgNPs formation was change the color of mixture from pale yellow to dark brown after adding $AgNo_3$. This color change could be noticed by nicked eye. As the color intensity increased, the accumulation of AgNPs increased (Elbeshehy *et al*., 2015), as appeared in figure (1). The extracellular biosynthesis of silver nanoparticles occurred during the exposure of the cell free extract (whey) to aqueous silver nitrate solution. The complete reduction of silver ions was observed 2-3h. The color change of the reaction was observed during the incubation period because the formation of silver nanoparticles is able to produce a specific color of the reaction mixtures because of their unique properties. The appearance of dark brown color is a conclusive indication of the formation of silver nanoparticles in the reaction mixture (figure 1). Due to the presence of coherent excitation of all the 'free' electrons within the conduction band, the color could be exhibited by the metal particles and leading to an in-phase oscillation which is known as surface plasmon resonance (SPR) (Maier, 2007).

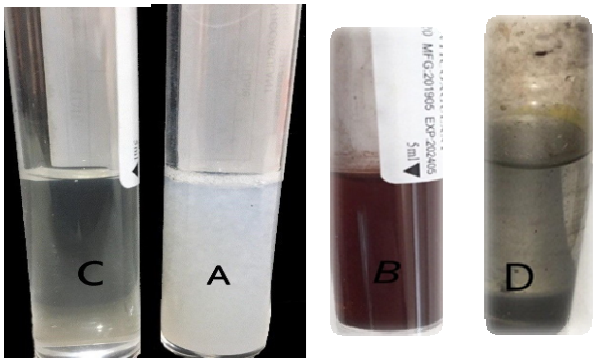

Fig. 1: Silver nanoparticle biosynthesis:
(A) The moment of adding silver nitrate to the whey.
(B) The color of the whey change to deep brown after 6 hr. after adding $AgNo_3$.
(D) The precipitate formed after 24 hrs. (C) The whey without $AgNo_3$ (control).

Characterization of Silver Nanoparticles

X-ray Diffraction (XRD) analysis

Pattern of X-Ray Diffraction measurements are used to examine structure intersystem of crystalline behavior of films at special condition.The result as shown in figure (2) was determined the presence of two types of nanomaterials Ag and AgCl in the sample, these results are consistent with the results (Zhao *et al*., 2015). The particle size of Ag and AgCl is calculated by Scherrer equation. The calculated particle size was ranged17.1-18.6 nm for Ag, and 24.3 nm for and AgCl.

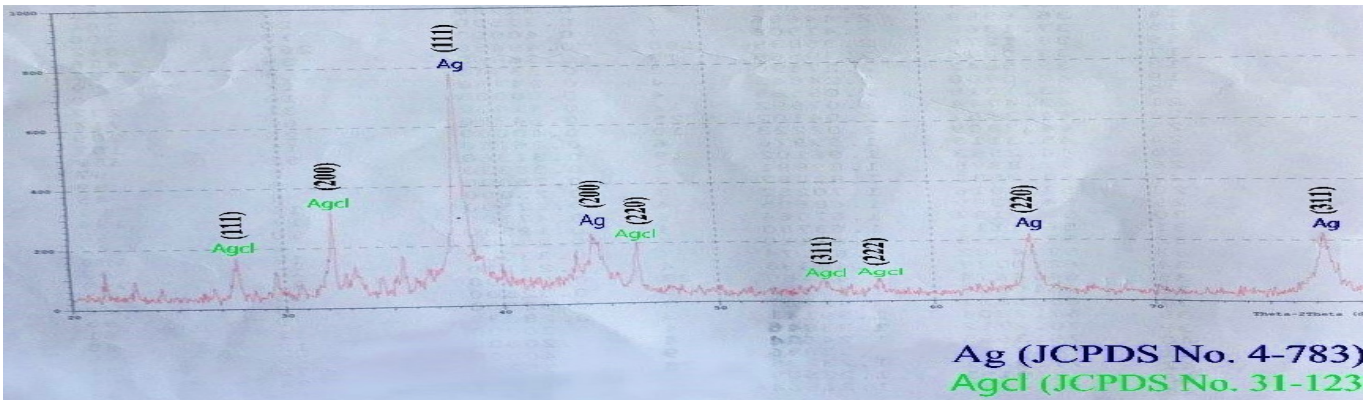

Fig. 2: X-ray Diffraction (XRD) analysis

Atomic Force Microscopy

The distribution sizetopography and granularity volume of nanoparticles were characterized by the use Atomic Force Microscopy (AFM). Average diameter were 50.09 nm, 35.00 nm, 50.00 nm≤50%, and 55.00 nm ≤90%.) 2D image and 3D image of Silver Nanoparticles synthesis figure (3).

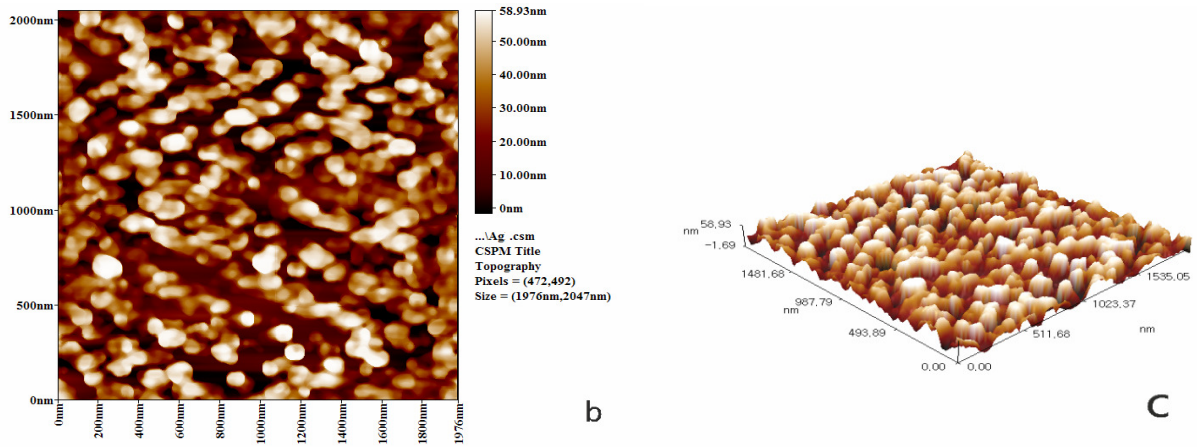

Fig. 3: Biosynthesized Ag/Agcl NPs by of Lactobacillus spp.
(b) 2D image of Silver Nanoparticles synthesis
(c) 3D image of Silver Nanoparticles synthesis

Scanning Electron Microscopy (SEM) analysis

Scanning electron microscopy (SEM) is a sort of electron magnifying lens that produce pictures of a sample by examining it with an engaged beam of electron. The electrons interface with particles in the specimen, creating different signs that can be distinguished and that contain data about the samples surface geology and organization, the nanoparticlesamples were scanned to saw the shape and size as well as the aggregation of the particlesas appeared in figure 4.

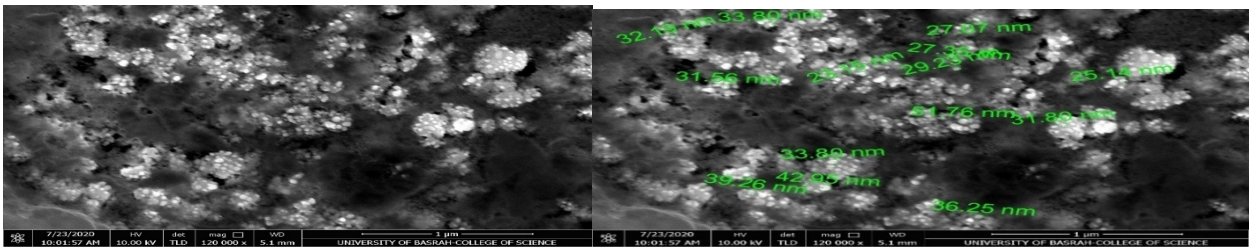

Fig. 4: The SEM images of Ag/Agcl nanoparticles

UV-Vis Spectroscopy

As cleared by Nayak *et al*. (2011) biological method of synthesis of silver nanoparticles exhibit strong absorption of electromagnetic waves in the visible range due to their optical resonant property, called Surface Plasmon Resonance (SPR). The SPR is highly influenced by shape and size of the nanoparticles. Due to the small size of silver nanoparticles in the quantum regime, the sample exhibited a stronger absorption at (400–650 nm). The strong absorption of visible light was responsible for the visible-light-driven photocatalytic activity of Ag/AgCl. The absorption band between (290-350 nm) could be attributed to the characteristic absorption of AgCl composite which possesses a direct band gap of 5.6 eV and an indirect band gap of 3.25 eV This matches with the study of Zhao *et al*. (2015).A study by Deljou and Goudarzi (2016) showed that silver nanoparticles biosynthesized by thermophilic *bacillus spp*. was at absorbance 425nm figure (5).

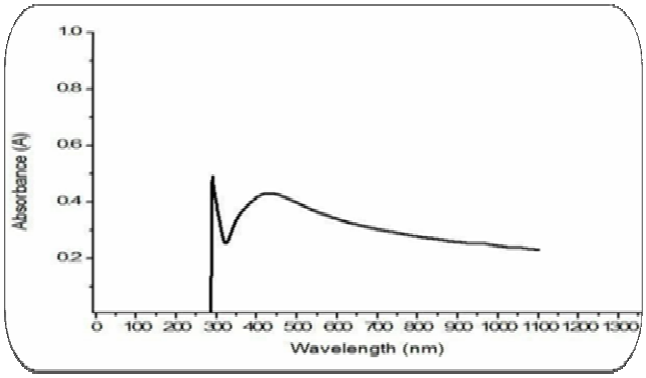

Fig. 5: UV-visible spectroscopy of synthesized Ag/AgCl NPs

Fourier-Transform Infrared Spectroscopy (FTIR) analysis

FTIR was performed to determine the possible functional groups of biomolecules involved in the reduction of silver and silver chloride ions and stabilization of the biosynthesized Ag/AgCl NPs from *Lactobacillus spp*. The FTIR spectrum of biosynthesized Ag/Agcl NPs showed eight distinct peaks (figure 6). The peak at 3381 cm^{-1} corresponded to asymmetrical and symmetrical N–H stretching vibration of the aromatic amine. The narrow peaks at 2927 cm^{-1} represents from C-O and the peak at 2366 cm^{-1} corresponded to N-H and C-H stretching. The peak at 1600 cm^{-1} corresponded to aromatic C=C bonds. The peaks at 1382 cm^{-1} and 1031 cm^{-1} assigned to NO_3 and C-N stretching of aromatic compound, respectively (Alhazmi, 2019). The peaks at 416 and 540 cm^{-1} represent from nanoparticles (Ali *et al*., 2015).

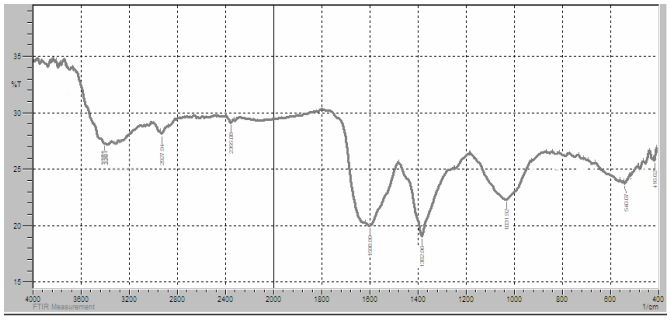

Fig. 6: FTIR analysis of Ag/Agcl nanoparticles

Determination the antibacterial activity of silver and silver chloride nanoparticles

Antibacterial activities of Ag and AgCl nanoparticles In our investigation have been evaluated against human pathogenic bacteria such as Gram-negative *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia, Proteus mirabilis;* the concentration of Silver nanoparticles (12.5mg/ml.) shown zones of inhibition 16 mm,19 mm, 18 mm and 21mm respectively while the diameter of inhibition zone raised to23 mm,24 mm,23 mm and 30mm against the same bacteria respectively when the concentration of the nanoparticles was 100mg/ml. The inhibitory effects of other concentration (25 and 50 mg/ml) of the nanoparticles were varied among the tested (gram negative) bacteria as shown in table 5 and figure 7.

On the other hand the effects of Ag and AgCl nanoparticles on the growth of gram positive isolated bacteria; *Staphylococcus aureus and staphylococcus epidermidis* were 24mm and 22mm at the concentration 12.5mg/ml respectively otherwise the diameter of inhibition zone recorded 32mm and 28mm at the concentration 100mg/ml against the same bacteria respectively, while the effects of the nanoparticles at the concentration 25mg/ml were 28mm and 24mm and at the concentration 50mg/ml were 30mm and 25mm against the same bacteria respectively as appeared in table 5 and figure 6.

Silver and silver chloride nanoparticles that biosynthesized from *Lactobacillus spp.* isolates have shown antimicrobial activity against the selected multiple drugs resist (MDR).The results of current study were following that obtained by Al-Tameme, (2017) who study the antibacterial activity of AgNPs against *P. aeuroginosa, S. aureus, E. coli*, *K. pneumoniae* and found the highest activity against *S. aureus* also another study by Kalishwaralal *et al*. (2010)who found that the anti-microbial activity of AgNPs against *P. aeruginosa* and *S. epidermidis*, is highest at the concentration of 100 µg/ml by using well-diffusion method. Generally the Gram positive pathogens were more susceptible to the SNPs compared to their Gram-negative. The antimicrobial properties of silver nanoparticles are well-established and several mechanisms for their bactericidal effects have been

proposed. Toxicity of the SNPs is presumed to be size and shape dependent because small size nanoparticles may pass through cell membranes. Inside a bacterium, nanoparticles can interact with DNA, thus losing its ability to replicate which may lead to the cell death (Hwang *et al*., 2012). Prabhu *et al.* (2014) also reported that *Staphylococcus aureus*, a Gram-positive organism, was the most susceptible to their Silver NPs.

Production of Silver NPs using a biological agent is a cost-effective and ecofriendly method. Synthesis of silver nanoparticles using a probiotic microbe (*Brevibacterium linens*) was reported by (Nithya and Ragunathan, 2012). Similarly, synthesis of Silver NPs by bacteriocin producing Lactobacillus species isolated from yoghurt was reported by (Prabhu *et al*., 2014). The traditional and modern use of LAB in industrial production is due to their many functional characteristics (Shihata and Shah, 2002).

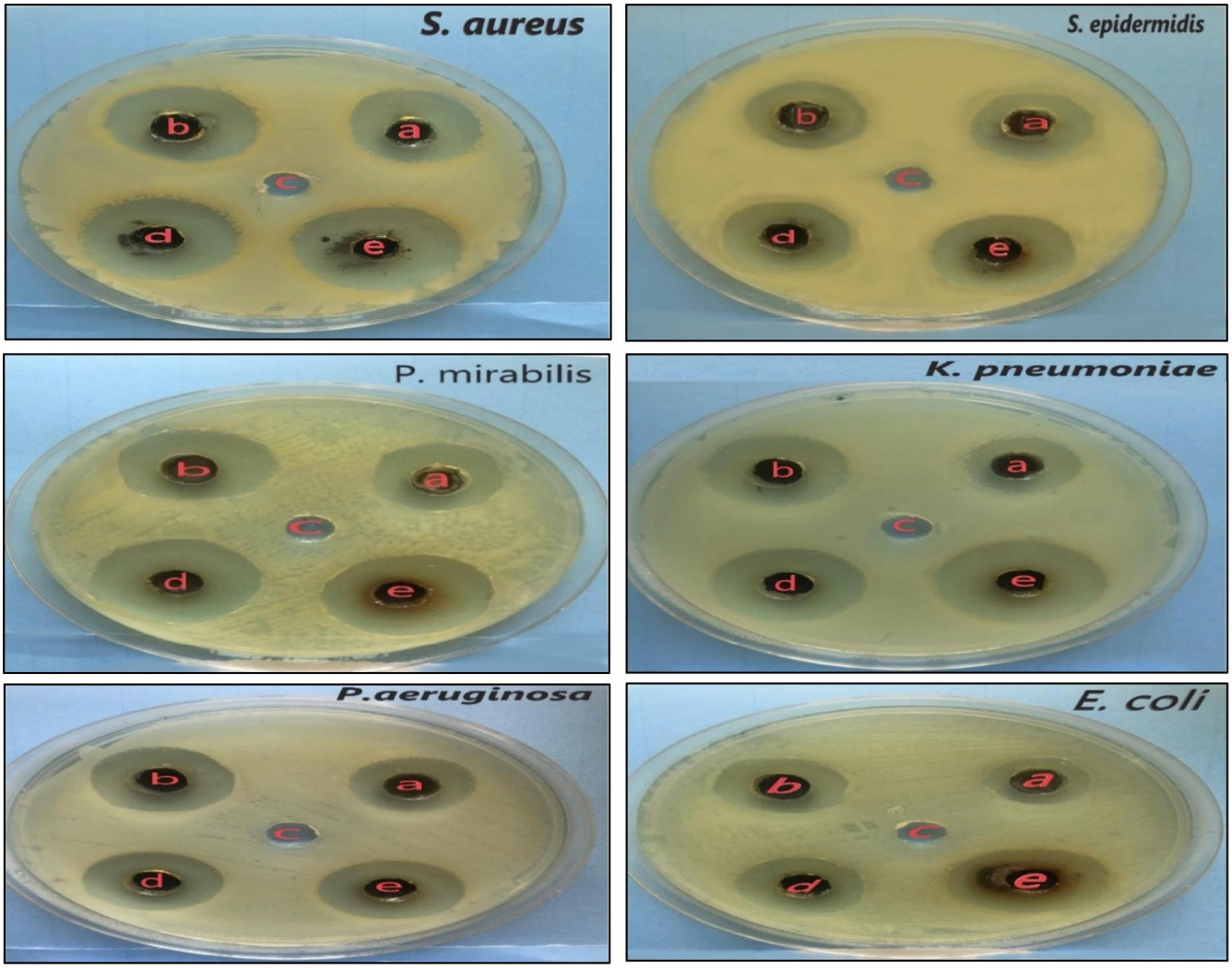

Fig. 7: Antibacterial activity of Ag/Agcl NPs against *S. aureus*, *S. epidermidis, P. mirabilis*, *K. pneumoniae, P. aeruginosa* and *E. coli* by agar well diffusion method. The letters (a, b, d, e,) represent the concentrations (12.5,25,50,100) mg/ml respectively, C (control).

Table 5: Antibacterial activity of Ag/Agcl nanoparticles at different concentration

Bacterial isolates	**Average. inhibition zone(mm) con. 12.5 mg/ml**	**Average. inhibition zone(mm) con. 25 mg/ml**	**Average. inhibition zone(mm) con.50 mg/ml**	**Average. Inhibition zone (mm) Con.100 mg/ml**
S. aureus	24 mm	28 mm	30 mm	32 mm
S. epidermidis	22 mm	24 mm	25 mm	28 mm
P. mirabilis	21 mm	24 mm	28 mm	30 mm
P. aeruginosa	19 mm	22 mm	24 mm	26 mm
E. coli	16 mm	21 mm	23 mm	27 mm
K. pneumoniae	18 mm	23 mm	25 mm	28 mm

Conclusion

The biosynthesis of Ag NPs by using the whey from local isolates of *Lactobacillus* spp. The calculated particle size was ranged17.1-18.6 nm for Ag, and 24.3 nm for and AgCl. The biosynthesized silver nanoparticles (Ag/AgCl NPs) have antibacterial activity against Gram-positive and Gram-negative bacteria.

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