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BIOSYNTHESIS OF SILVER NANOPARTICLES USING *Saccharomyces boulardii* AND STUDY THEIR BIOLOGICAL ACTIVITIES

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ABSTRACT

Objective: The objective of the present study is the biosynthesis of silver nanoparticles using probiotic, *Saccharomyces boulardii* and evaluating their biomedical activity. The biological method of nanoparticles synthesis acquires very important area due to their cost-effective and eco-friendly benefits over chemical and physical methods of synthesis. **Methods:** AgNPs were biosynthesized by adding silver nitrate ($AgNO_3$) into cell free supernatant of *S.boulardii* at concentration (10 mM). **Results:** The PCR was accomplished to molecular identification of *S.boulardii* using specific primers for (CAG) microsatellite repeat at locus YLR177w, foreword primer (Boul 3) and reverse primer (Boul 4). Biosynthesis of AgNPs was firstly indicated by the colour alteration of reaction mixture from yellow into reddish brown. The characterization completed by XRD, SEM, EDS and AFM. The XRD presented the size of AgNPs was 19nm.The SEM was offered the shape was spherical and homogenous and the size was ranged between 63-100nm. The occurrence of elemental silver was analysed by EDS. The AFM displayed the three dimensional structure of silver nanoparticles and the average diameter was 97.44 nm. Biosynthesized AgNPs showed antibacterial activity against multidrug resistant bacteria(MDR) of both gram positive and gram negative bacteria (*S.aureus, S.pyogenes, E.coli, K.pneumoniae, E.aerogens, S.typhi, A.baumannii, P.auroginosa and P.mirabilis*). All tested bacterial isolates revealed their ability to form biofilm in the form of biofilm using tube and Congo red method without treated by the nanoparticles except *P.mirabils, A.boumanii* and *E.aerogenes,*but when treated with AgNPs this ability was prevented and removed in *K.pneumoniae, Ps.auroginosa* and *C.neoformans*. The AgNPs at two concentration 1mg/ml and 2mg/ml bared their antioxidant capacity *in vitro* by scavenging DPPH free radicles, the largest inhibition titer found in the mixture of DPPH with biogenic AgNPs and supernatant of *S.boulardii* at concentration 2mg/ml (87.43).

KEYWORDS: Biosynthesis AgNPs, *S.boulardii*, Antibiofilm, Antimicrobial, Antioxidant Activity.

INTRODUCTION

Nanotechnology is field deals with production and use of Nanoparticles with 1-100nm. Nanoparticles have been considered because of their exclusive physicochemical individualities comprising antibacterial, catalytic, optical, electronic and magnetic properties.[1,2]

Ag nanoparticles have established attention because of their antimicrobial action and inhibition the biofilm creation, as well as their physicochemical, biological possessions and their requests in biomedicine, electronics and optics.[3,4]

There are sum of physical, chemical, biological, and hybrid procedures for making of nanoparticles. The first and second are more costly, energy and toxic.[5] Expansion of dependable, nontoxic, and green methods for production of nanoparticles is the essential to multiply their biomedical usages. Achieve this goal is to use microorganisms to synthesize nanoparticles.[6,7]

The extracellular creation of silver nanoparticles using yeasts species actions to be adequate to many uses,[8,9] and to improve new real antimicrobial and antioxidant means that overcome the MDR microorganisms,[10] Therefore the present study has been designed to biosynthesis of silver nanoparticles using *S.boulardii* species and studies their antimicrobial and antioxidant activity.

EXPERIMENTAL

Preparation supernatant of *S.boulardii*

S.boulardii was carefully chosen from numerous different bacterial species according to their resistance to commercial AgNP and their capability to extracellular fabrication (supernatant) to AgNP (data not shown). BHI was inoculated with *S.boulardii* and incubated at 37°C for 24 hrs. Colonies were handpicked and long-established as *S.boulardii* dependent on morphological, biochemical tests and PCR.[11]

The culture was centrifuged at 6000 rpm for 25 min, 4°C to make supernatant from *S.boulardii*. Cell free supernatants were composed for using in the biosynthesis of silver nanoparticle.[12]

Molecular Identification of *P.agglomerans*

Extraction of DNA from *S.boulardii* was done according to the kit FavorPrep Genomic DNA Extraction Mini Kit Favorgen / Korea. The concentration and purification of DNA was determined according to.[13]

The PCR was done to identify *S.boulardii* using specific primers for (CAG).[9] microsatellite repeat at locus YLR177w, foreword primer (Boul3) 5`CTTAAACAACAGCTCCCAAA 3` and reverse primer (Boul4) 5`ATTTCTGATGCGCTGATTCAT 3`.

A PCR mix was 2.5 ul (10µM) of forward and reverse primers, with tube of Accupower ®PCR-Pre Mix-Kit ((1unit of Top DNA polymerase, 250 mM Each: dNTP (dATP, dCTP, dGTP, dTTP), 10 mM Tris-HCl (pH 9.0), 30 mM KCl, 1.5 mM $MgCl_2$, Stabilizer and tracking dye)), and 8µL (50ng) of DNA template, volume was adjusted to 20ul of DW.

The reaction was run at 94 °C for 5 min followed by 35 cycles of 1min at 94 °C, 1min at 56 °C, and 2min at 72 °C and 7min at 72°C in a thermocycler. PCR Products (1500 bp) were electrophoresed on a 1.5% agarose gel stained ethidium bromide and photographed under Ultraviolet transilluminator.

Biosynthesis of silver nanoparticles using cell free supernatant

$AgNO_3$ was as ancestor for biosynthesis of silver nanoparticles by *S.boulardii.* $AgNO_3$ was added with concentration (1, 3, 5and 10 mM) to cell free supernatant which mixed. This step was organised in dark condition to avoid oxidation of $AgNO_3$.The pH of the reaction mix was adjusted to 8. The resultant solutions were incubated in shaking incubator 150 rpm at 37° C for 24 hrs.

After incubation the colour variation was noticed and the reaction mix was centrifuged at 6000 rpm for 25 min,4°C, the supernatant was unwanted and substituted with deionized distil water and re centrifuged three times at the same conditions to remove remained supernatant, the pellet characterize collection of nanoparticles was dried in oven at 40°C for 18-24 hours. The dried powder was collected carefully and stored for further analysis.[14,15]

Characterization of silver nanoparticles

The X-ray diffraction was used for characterization of silver nanoparticle. SEM (Inspect S50. FEI) was used for characterization the morphology of nanoparticles in electron microscope unit. The microscope operated at an accelerated voltage at 15 KV and different magnification, low vacuum, a spot size 4 and working distances5-10mm.[16] Elemental analysis of single particle was carried out using Bruker EDS attached with SEM. EDS performed for point analysis with accelerating voltage 10 KV, spote size 5, working distances 10mm, this analysis was used to detect presence of elements nanoparticles.[17] Atomic force microscope (AFM) was used for characterization the silver nanoparticle.

Antibacterial activity of nanoparticles

Antibacterial activity of biogenic AgNPs was carried by agar well diffusion against different kinds of pathogenic multidrug resistant bacteria of both gram positive and gram negative(table 1).Standardized suspension of each tested bacteria ($1.5x10^8$cfu/ml) by McFarland standard (0.5N) then swabbed separately onto sterile Muller-Hinton Agar (MHA) plates using sterile cotton swabs.

Agar was punched with sterilized cork borer 6 mm and 100µl (150µg/ml) from commercial SNPs and biogenic AgNPs was added into each well, incubated for 24 hrs at 37°C, after incubation the inhibition zones were measured.[18] number of bacteria used as indicator strains, *Acinetobacter baumannii, Enterobacter aerogens ,Escherichia coli ,Klebseilla pneumonia ,Proteus mirabilis Pseudomonas aeruginosa ,Salmonella typhi ,Staphylcoccus aureus ,Steptococcus pyogenes*.

Antibiofilm activity of silver nanoparticles

Tube and Congo red agar methods were used for qualitative valuation of biofilm formation and antibiofilm activity by nanoparticles as described by,[19,20]

Antioxidant activity of biogeneic silver nanoparticles in vitro

DPPH is a free radical scavenging assay was used to evaluate the ability of the commercial AgNPs and biogenic AgNPs annihilate the DPPH free radical. The process refer to by[21] with some modification was used. The biogenic AgNPs were added to DPPH (0.1mM) at concentration 1 and 2mg/ml, 0.5ml of both supernatant and microorganism growth of *S. boulardii* and *P. agglomerans* were added to 2ml of metabolic solution of DPPH (0.1mM).

The reaction mixture was incubated for 30 min in dark room at 37°C and the absorbance (A) was read at 517 nm in spectrophotometer. The experiment was repeated for three times. DPPH solution was used as a control (without sample) and ethanol 99.8% as blank. The inhibition of the DPPH radical by biogenic AgNPs was calculated according to the following formula:

% of Inhibition = ((Abs of control – Abs of test))/(Abs of control) X100.

RESULTS

Biosynthesis of Silver nanoparticles

S.boulardii exhibited their ability in the extracellular biosynthesis of AGNPS using cell free supernatant and AgNO3 (10Mm) as a precursor. After shaking incubation

for 24 hrs at 37°C at 150rpm, *S.boulardii* have the ability in changing the colour of reaction mixture from yellow to reddish brown which denotes as indicator for biosynthesis the AGNPS.

Identification by PCR

Result revealed the single band with 130bp (fig1)

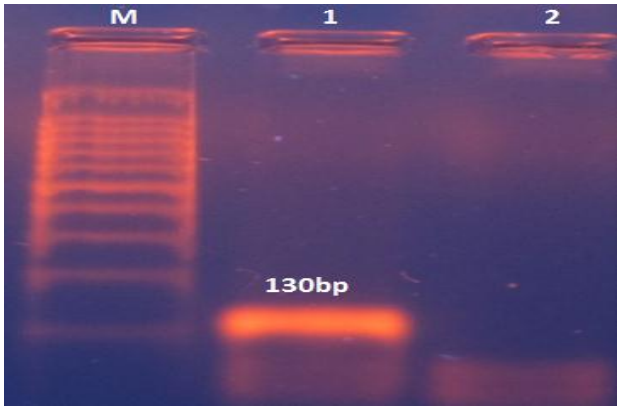


Fig. 1: Agarose gel electrophoresis (1.5% Agarose gel, 75 volts to 1 hours) for PCR product (130bp) of $(CAG)_9$ microsatellite repeat at locus YLR177w of *S.boulardii* Lane (M) :size marker (200 bp DNA Even Ladder); Lane 1 PCR product, Lane 2: negative control.

XRD analysis of nanoparticles

XRD showed that, *S.boulardii* produced silver nanoparticles with average size 19nm (fig.2).

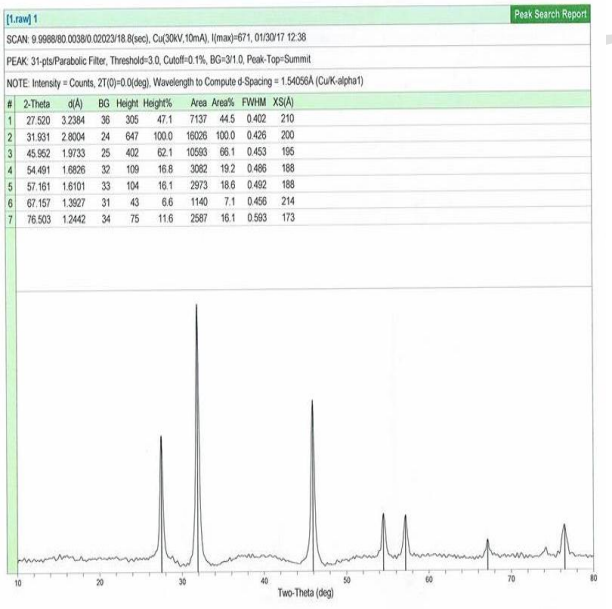


Fig. 2: XRD analysis of Biosynthesized nanoparticles using S.boulardii (average diameter 19.92nm).

SEM analysis of nanoparticles

SEM results showed well-dispersed nanoparticles and homogenous with diameter of 60-100nm for AgNPs, with variable shapes most of them present in spherical form (fig 3).

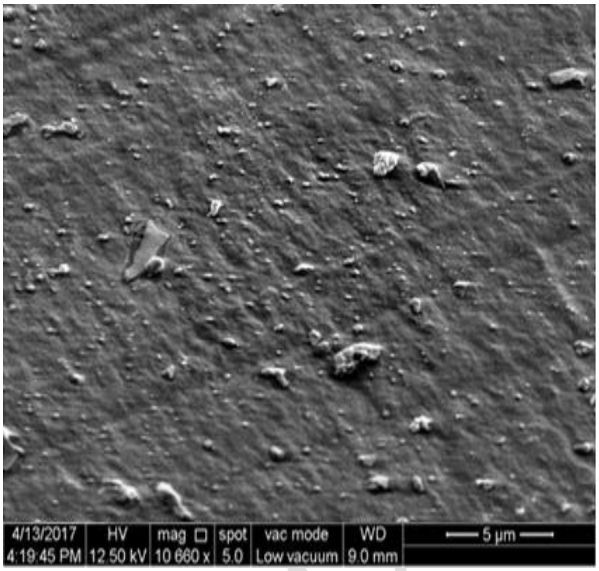
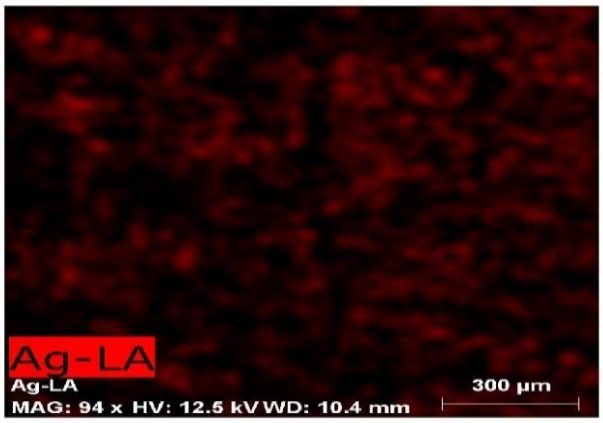
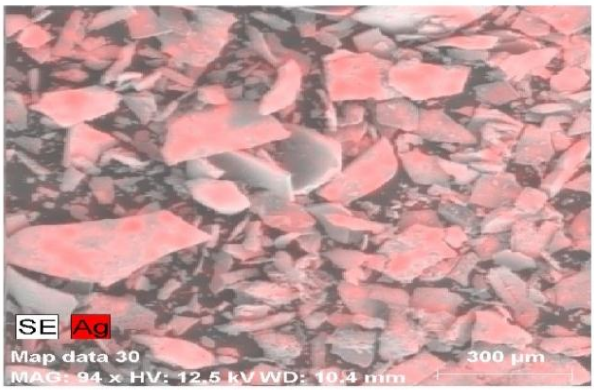


Fig. 3: SEM micrograph of biogenic silver nanoparticles. The shape of AgNPs was spherical and homogenous, size between (63-100nm).

EDS analysis of nanoparticles

The presence of elemental silver designated the reduction of silver ions in mix by *S.boulardii* supernatant. The EDS spectrum was recorded in the point and map mode, strong signals from the Ag atoms were observed while medium signals from oxygen and weaker signals from other atoms. The weight percentage of elemental constituents for AgNPs that was92.71% silver (fig.4).





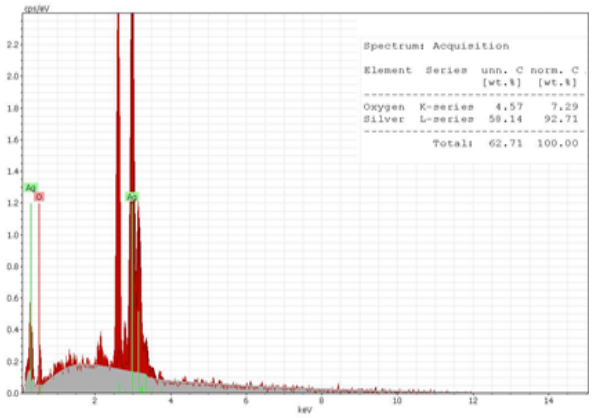
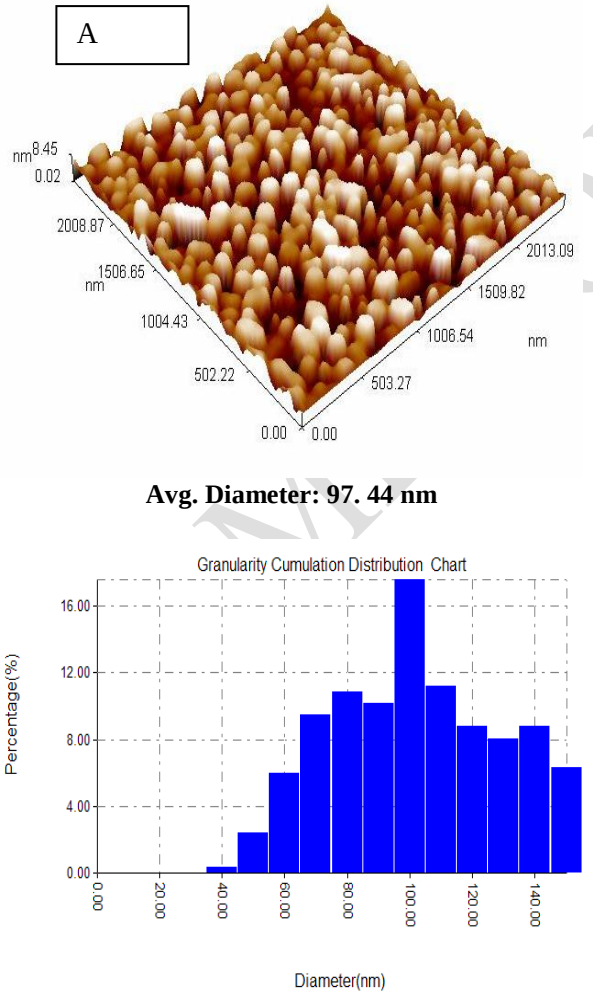


Fig. 4: EDS analysis (point and mapping) of biogenic silver nanoparticles: Illustrated strong signals from the Ag, medium signal from O_2, the optical absorption peak of Ag was observed at 3Kev, the weight percentage of silver (92.71%).

AFM analysis of nanoparticles

(AFM) analysis seemed, *S.boulardii* produced AgNPs with average diameter 97.44% (fig.5).



Avg. Diameter: 97. 44 nm

Fig. 5: Atomic Force Microscopic analysis of biosynthesized silver nanoparticle from *S.boulardii* (Average Diameter: 97.44 nm).

A-three dimension of nanoparticles
B-Granularity cumulation distribution chart of nanoparticles

Antibacterial activity

Results showed that AgNPs has the ability to inhibit the bacterial growth gram positive and gram negative bacteria. The inhibition zone was greater in gram negative than in gram positive bacteria. The largest inhibition zone of commercial AgNPs in Gram negative bacteria was 15mm in *Ps. auroginosa* with concentration 150µg/ml**,** while the largest inhibition zone in Gram positive bacteria was 16mm in *Streptococcus* with same concentration, while the SNPs synthesized from *S.boulardii* showed large inhibition zone in Gram negative bacteria which was 29 mm in *Ps.auroginosa* ;and large inhibition zone in Gram positive bacteria was 29mm in *S.pyogen* at the same concentration 150ug/ml in addition the antibacterial activity in other bacteria were also different in their sensitivity to AgNPs when exposed to the same concentration (table 1).

Table 1: Inhibition Zones of pathogenic bacteria in mm by AgNPs at concentration 150ug/ml.

Tested microorganisms	Commercial AgNPs	AgNPs *S.boulardii*
A.baumannii	0	23
A.fumigatus	0	0
A.niger	0	0
C.neoformans	0	0
E.aerogenes	11	16
E.coli	14	21
K.pneumoniae	0	20
p. aeruginosa	15	29
P. agglomerans	0	17
P.chrysogenium	0	0
P.mirabilis	0	16
S.aureus	0	18
S.pyogen	16	29
S.typhi	18	21

Antibiofilm activity of silver nanoparticles congo red method

The experiment was tested 12 isolates two of them was not producing biofilm (*E.aerogenes and P.mirablis*), the biogenic AgNPs from *S.boulardii* was prevented the formation of biofilm in many isolates such as *A.boumanii and E.coli* while some isolates was inhibited but not preventing the formation of biofilm such as *C.neoformans* and *K.pneumonia* also but the biogenic AgNPs not have effect on the formation of biofilm in *S.aureas* (table 2).

Table 2: Biofilm activity using congo red method for different microorgansims.

Microorganism	Without AgNPs	Commercial AgNPs	Biogenic AgNPs from *S.boulardii*
A.boumanii	Weak	Weak	None
C.neoformans	Moderate	Moderate	Weak
E.aerogenes	None	None	None
E.coli	Moderate	Strong	None
K.pneumonia	Strong	Modetera	Moderate
P.agglomerans	Strong	moderate	Weak
P.mirabils	None	None	None
Ps.auroginosa	Moderate	Weak	Weak
S.aureas	Weak	Weak	Weak
S.boulardii	Strong	Moderate	Weak
S.pyogen	Weak	Moderate	Weak
S.typhi	Weak	Moderate	Weak

Tube method

Twelve isolates was used, two of them was not producing biofilm (*E.aerogenes and P.mirablis*), the biogenic AgNPs from *S.boulardii* was prevented the formation of biofilm in many isolates such as *C.neoformans* and *K.pneumonia* while some isolates is inhibited but not preventing the formation of biofilm such as *S.aureas* and *S.typhi* (table 3).

Table 3: Biofilm activity using tube method for different microorganisms.

Tested microorganism	Without AgNPs	Commercial AgNPs	Biogenic AgNPs from *S.boulardii*
A.boumanii	moderate	Weak	None
C.neoformans	Strong	Strong	None
E.aerogenes	none	None	None
E.coli	Weak	Strong	None
K.pneumonia	moderate	Strong	None
P.agglomerans	moderate	Weak	None
P.mirabils	None	None	None
Ps.auroginosa	Strong	Strong	None
S.aureas	Strong	Moderate	Weak
S.boulardii	Strong	Moderate	None
S.pyogen	Strong	Moderate	None
S.typhi	Weak	Strong	Weak

Antioxidant activity of biogenic AgNPs nanoparticles

After adding the nanoparticles and *S. boulardii* to DPPH solution (0.1M).The results revealed ability of nanoparticles and *S. boulardii* to scavenging DPPH free radicles that indicated by observing the colour change from the original colour of DPPH purple into yellow colour.

These results demonstrated the antioxidant activity of biosynthesized AgNPs and *S. boulardii in vitro* and led to evaluation the competency of nanoparticles and *S. boulardii* for antioxidant activity *in vitro*. The largest inhibition titer found in the mixture of DPPH with biogenic AgNPs and supernatant of *S.boulardii* at concentration 2mg/ml (87.43).

DISCUSSION

The bacteria which have the "Silver resistance machinery" can make AgNPs. Extracts from bacteria may do both as reducing and capping means in AgNPs creation. The reduction of silver ions by mixtures of biomolecules such as enzymes/proteins, amino acids, polysaccharides and vitamins is environmentally benign and chemically complex.[22]

The appearance of a brown color in solution is an indication of the development of AgNPs in the mixture due to reduction of Ag+ ions to Ag metal by the reducing agents such as enzymes, proteins, amino acid, polysaccharides etc.[23,24,25]

The environment of the culture supernatant can be simply enhanced than the cell, where the ingredients in the cytoplasm would try to keep constant environment and necessitate more purification Therefore, supernatant can be used for the creation of silver nanoparticles rather than cells itself.[26]

A numeral of studies directed that NADH and NADH-dependent enzymes are factors in the biosynthesis of metal nanoparticles. The reduction looks to be started by electron transfer from the NADH by NADH-dependent reductase as electron carrier.[27,28]

The morphology are dependent on several chemical and physical parameters, e.g., incubation time, pH, composition of the culture medium and growth in the light or dark.[29] Shape and size organised nanoparticles could be manufactured by modifying the pH or the temp of the reaction mix.[30] at 65Cº, fewer quantities of NPs were produced, whereas at 35Cº more quantity of NPs were made. At acidic pH, the AgNPs synthesis reduced due to the alkaline ion (-OH) is very much requisite for the reduction of metal ions there will be a smaller amount nucleation for silver crystal creation on which new entering silver atoms deposit to form larger sized particles. While as the pH upturn towards alkaline region, the dynamics of the ions and production improves and ranges the maximum at pH 10 and extra nucleation regions are made due to the accessibility of –OH ions. The alteration of Ag+ to Ag0 rises followed by increase in the kinetics of the deposition of the silver atoms.[31]

XRD analysis detected the average size of silver nanoparticle; the AgNPs biosynthesized from *S.boulardii* was 19.92 nm and this size is suitable in contrast with other studies.[32]

SEM used to define the shape and size of biogenic nanoparticles, trial results exhibited well-dispersed nanoparticles with diameter of 63-100nm for silver nanoparticles biosynthesized using *S.boulardii* with variable shapes.[33,34]

EDS analysis revealed the occurrence of elemental silver which directed the reduction of silver ions to silver medals in the reaction mixture, the weight percentage of silver was 92.71% for AgNPs biosynthesized from *S.boulardii*. The optical absorption peak was observed at 3keV which is a typical absorption of metallic AgNPs.[35,36]

AFM analysis presented the 3 dimensional shape of silver nanoparticle and the average diameter of the nanoparticle the average diameter of silver nanoparticle biosynthesized from *S.boulardii* was 97.44 nm.

AgNPs amalgamation was better in term of quality; minimum size and less polydispersity, with S. boulardii, this result may be attributed to the differences in the bio-reduction that may be return to the qualitative and quantitative of extracellular protein/enzyme and other biomolecules that offered in the culture of each microorganism, in addition to their ability of interaction with $AgNO_3$.[37]

Nanoparticles are a viable alternative to antibiotics and seem to have a high potential to solve this problem. AgNPs were considered particularly attractive for the production of a new class of antimicrobials.[38,39,40]

The AgNPs looked their antibacterial effects on gram positive and gram negative bacteria. The largest inhibition zone was exposed in G-ve in evaluation with G+ve bacteria, the maximum inhibition zone of commercial AgNPs in G-ve was (18mm) in *S.typhi* with concentration (150µg/ml) while the maximum inhibition zone in G+ve was (16mm) in *S. pyogen* with the same concentration, while for AgNPs biosynthesized from *S.boulardii* in G-ve was (29mm) in *Ps. auroginosa* while in G+ve was (29mm) in *S. pyogen*[41,42,43]

Also there is variances in the sensitivity of tested pathogenic bacteria to AgNPs when exposed to the same concentration(150µg/ml) of commercial AgNPs such as *E.coli*, *Ps.aeruginosa and E.aerogenes*, the inhibition zone of these bacteria was (14,15 and11 mm) respectively, while the AgNPs biosynthesized from *S.boulardii* was (21,29,16mm) this may be return to the differences in intrinsic exposure of bacterial species depends on the concerted activity of several elements, what has been named as intrinsic resistome.[44,45]

When rise the concentration of silver nanoparticles presented rise in the antibacterial activity, these results similar with.[46,47] The antimicrobial effect due to The positive charge on the silver ion as it can interest the negatively charged of microorganisms through the electrostatic interaction. This attraction probably overcomes other factors, such as size and shape that can influence the bacterial cell death.[48,49,50]

pH-Dependent biosynthesized AgNPs have individual role in the antibacterial activity by these nanoparticles, the smallest nanoparticles created in alkaline pH demonstrated more antibacterial activity than the large particles which are synthesized in acidic pH.[51]

The shape of nanoparticles play very significant role in the antimicrobial activity of nanoparticles.[52;53] Silver ions bind to nucleic acid and protein negatively charge causing deformation and structural changes in the cell wall, in the membrane and in the nucleic acids of bacterial cells. Silver ion interacts with a number of electron donor functional groups such as phosphates, thiols, hydroxyls, indoles and imidazoles. The AgNPs also damage membranes and induce the release of reactive oxygen species (ROS), forming free radicals with a powerful bactericidal action.[54]

The silver ions is known to mainly inhibit enzymes such as NADH dehydrogenase II in the respiratory system, which is involved as a candidate for the site of production of reactive oxygen species.[55] Small sized nanoparticles exhibited more antibacterial activity than large size particles because the small sized particles effect on a large surface area of the bacteria.[56,57,58]

It has been suggested that AgNPs interfere with bacterial replication processes by adhering to their nucleic acids. The interaction of silver ions with sulfhydryl (–SH) groups of proteins that origin the DNA unwinding and

contact with hydrogen bonding processes are also been established lead to cell division was inhibited.[59,60]

The ribosomes may be denatured by silver ions or small AgNPs as a consequence inhibition of protein synthesis as well as translation and transcription can be blocked by the binding the AgNPs with the genetic material of the bacterial cell.[61,62]

It has also been found that the nanoparticles can modulate the signal transduction in bacteria by dephosphorylate the peptide substrates on tyrosine residues, which leads to signal transduction inhibition and thus the stoppage of growth.[63,64]

Biofilms are complex bacterial populations that resist the action of antibiotics and the human immune system. Due to the lack of effective antibiofilm antibiotics. Nanoparticles were used to resolve this problem, one potentially important candidate treatment uses AgNPs to show anti-biofilm activity.[65,66]

All tested microorganisms showed their ability to form biofilm in the form of film lined the wall and bottom of tubes in the tube method without treated by the nanoparticles except *P.mirabils*, *A.boumanii* and *E.aerogenes*, but when treated with AgNPs this ability was prevented and removed in *K.pneumoniae*, *Ps.auroginosa* and *C.neoformans*, AgNPs may be altered gene the expression relating to biofilm formation, as consequence they effect on microcolony formation and biofilm maturation. This lead to AgNPs could be used for prevention and treatment of biofilm-related infections.[67,68,69]

The antibiofilm activity of AgNPs was observed less effective against G+ve bacteria than on that of G-ve bacteria this remark may be a result of the structural differences in the composition of the cell wall in G+ve and G-ve bacteria.[70] other study revealed that AgNPs have antibiofilm ability against G+ve and G-ve bacteria when catheters coated with AgNPs were tested *in vitro* observed almost complete prevention of biofilm formation by *E.coli, S.aureus* and *C. albicans*.[71]

Generated hydroxyl radicals can depolymerize polysaccharides, cause breaks in DNA and inactivate enzymes that can compromise the EPS matrix of the biofilm architecture.[72,73]

The cause of remaining the biofilm in some tested bacteria such as *S.typhi and S. aureus* may be due to resistance of bacterial strain to AgNPs, some strains within a given species may be sensitive and others may be resistant or to the size of nanoparticles that may be used.[74,75,76]

DPPH is a more stable and well-known free radical based on the reduction of accepting hydrogen or electron from donors. The DPPH reducing ability of the antioxidants (AgNPs and *S.boulardii*) were evaluated by seeing colour change from original deep purple colour of DPPH into yellow colour after adding AgNPs in addition to the growth suspension and supernatant of *S.boulardii* to DPPH.

DPPH scavenging activity of nanoparticles better with increasing their concentration that showed by the elevated percentage of inhibition of DPPH which increased with increase concentration of AgNPs that exhibited more inhibition (72.67% in1mg/ml) and (22.95% in 2 mg/ml) for AgNPs from *S. boulardii* respectively due to more an electron donated and accepts by DPPH.[77,78]

The inhibition proportion by bacterial suspension of *S.boulardii* was (12.56%) and cell free supernatant was 25.13%. The diverse mechanisms involved in the radical-antioxidant reactions may explain the different in scavenging potentials of the compounds. The mechanisms of antioxidants are not only by scavenging free radicals, but also by inhibiting production of free radicals.[79,80]

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