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Antibacterial and Antibiofilm Activity of Bacteria Mediated Synthesized Fe_3O_4 nanoparticles Using *Bacillus Coagulans*

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ABSTRACT

There are several types of nanoparticles, and it may be that metals and their oxides have been important since ancient times for medical uses, Iron Oxide NPs was preferred among all for its unique properties, supernatant of gram positive Bacillus Coagulans bacteria was employed in synthesis of IONPs as stabilizing and bio reducing agent, the synthesized iron oxide NPs were characterized by Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD),Scanning electron microscope (SEM) and Atomic force microscope(AFM) ,the short incubation time and rapid precipitated in the synthesis protocol of NPs resulting in fine agglomeration and small size of particles were calculated from (XRD) in avenge about 15.2 nm , and to reveal the activity of IONPs as an antibacterial and antibiofilm formation agent respectively, it was experimented against Uropathogenic E.coli bacteria isolated from urinary tract infection (UTIs) patients, by well diffusion method and it was exhibited potent antibacterial activity in depends on concentration ,also Fe_3O_4 NPs was displayed strong antibiofilm activity were test in concentration of MIC at 150 µg/ml through two methods, congo red and tube method .

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INTRODUCTION

Biofilm formation by E. coli and others pathogenic bacteria plays an important role in reducing the effectiveness of antibiotic and the persistence of pathogenesis by giving the character of resistance to those bacteria[1], the thickens of E. coli biofilm and presence of exopolymers make it difficult to penetrated by host immune cells and treatment[2], the building of matrix and the accumulation of different material give advantages to biofilm in up to 1000-fold to resistant to conventional antibiotic from planktonic cells[3], due to several mechanism like, limitations of antibiotic diffusion through the matrix[4], Transmission of resistant genes within community[5,6] , Expression of efflux pumps[7],lower cell permeability[8], in addition to that there is enzymes interference to modified and proteins to neutralized drug [9,10], other mechanism of antibiotic resistant in biofilm as well as in the plankton, Inactivation of the antibiotic by change in concentration of metal ions and pH values[11], one of the alternative ways to eliminate pathogenic bacteria and reduce the chances of

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developing biofilm formation through the use of nanoparticles[12,13],many types of NPs have ability to cross and penetration biological barriers were disruption the community of bacterial biofilm[14,15],and the most winning is metal oxide nanoparticles which recorded victories in their battle against resistant bacteria[16], and biofilm formation[17], as a result of the appropriate characteristics such as size, shape, roughness and surface area [18]. There are Three methods to synthesis nanoparticles, chemical, physical, and biological[19], although chemical and physical methods widely use in production and limits used in biomedical application duo to their possible toxicity or release hazard compounds more over consumption large amount of energy[20]. Hence, based on versatile application in biomedicine encouraged the researcher to improvement and development of Bio-assisted methods in nanomaterial production .[21].otherwise, biosynthesis provided environmentally benign, cost-effective, low toxic, efficient protocol to synthesis and fabricated in size and shape of nanoparticles [22], biosynthesis method broadly using bacteria, algae, yeast [23,24,25], and plant extracted [26].

Fe_3O_4 NPs one of most important nanoparticles used in biomedical application[27], due to unique properties such as biocompatibility[28], superparamagntic behavior[29],structure mode, chemically stable and easy synergistic or coated with organic and inorganic materials [30], rapid and easy separation by using external magnetic field, no toxic waste in biosynthesis and powerful in scalable[31], high surface area to volume[32]. also this properties provided other auxiliary power for success the iron oxide NPs in bactericidal effectiveness against antibiotic-resistant bacteria. [33] and biofilm formation[34], those IONPs was experimented for antimicrobial activity against Uropathogenic E. coli ,also antibiofilm formation was investigated by congo red and tube method for same bacteria.

MATERIALS AND METHODS

Chemicals

Chemical reagents, Iron (II) chloride tetrahydrate ($FeCl_2.4H_2O$,99%) and Iron (III) chloride hexahydrate ($Fe_3Cl.6H_2O$,97%) were purchased from THOMAS BARKER (India),and sodium hydroxide (NaOH) was supplied from HIMEDIA (India). All reagents was used without further purification and the aqueous solution were prepared using double deionized water while the glassware used was cleaned and sterilized following standard laboratory procedure.

Bacterial identification

The bacteria B .coagulans were obtained from biology department, college of science, Babylon University, Iraq the bacteria colonies grown in brain heart infusion agar was frosted glass, cream light yellow appearance but may become opaque or smooth raised wrinkly colonies. While the microscope examination of the bacteria smear was showed , gram positive rods, appear in chains or pairs, spore forming, and the single spore were ellipsoidal in shape, subterminally to Paracentrally located, take light green color when stain with malachite green . in addition, bacteria B. coagulans are motile and capable of producing lactic acid.

Bacteria culture collection

The supernatant of B. coagulans was collected to be used in synthesis of IONPs through bacteria grown in Brain heart infusion broth medium, the BHI broth in 250 ml conical flask was incubated at 37 °C for 24 hr. on shaker to mixed homogenously, after incubation the bacteria broth culture was centrifuge for 10 minutes at 10000 rpm, finally the upper free cell supernatant layer was collected to use in further step of Fe_3O_4 NPs biosynthesis.

Synthesis of Iron oxide NPs

Supernatant of bacteria B. coagulans was used in synthesis of Fe_3O_4 NPs as stabilizing and capping agent in simple co-precipitated method, with slight modification, the iron salt precursor Fe^{3+} and Fe^{2+} at a 2:1 M ratio was added to aqueous solution(supernatant) to dissolved and mixing by using magnetic stirrer at 35°C . Simultaneously, NaOH 1.0 M which was prepared freshly were added in dropwise into stirring solution to adjust the pH ~11.the changing in color solution (Fig.1) becoming dark and more precipitate appear with time among stirring for 30 minutes, the synthesized IONPs was collection after completion of reaction using external magnet, washed three time with deionized water, finally dried in an oven over night at 70 °C for further characteristics[35].

Characterization techniques

x-ray diffraction

XRD analyses was perform to reveal the

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crystallographic structure and estimated average size of synthesized iron oxide nanoparticles from scherrer equation as $Ds=K\lambda / \beta Cos\theta$ were Ds is average of particles size, K is equal to 0.9 as scherrer constant value, λ is the wave length of CuKα irradiation ,x-ray Tube; Cu(1.5406 A^o) .β is a full width at the half maximum intensity (FWHM) of the diffraction peak and θ is the diffraction angel of the peak . The crystalline nature of synthesis IONPs confirm with XRD analysis as well as the average size (15.13 nm) of NPs within nanoscale and the size of the nanoparticles may appear small, and this may be a result of the reaction rate due to the presence of NaOH and biomolecules in the supernatant.

Scanning electron microscope(SEM)

Scanning electron microscope (SEM) analysis clearly shown through images of nanoparticles the shape , morphology of synthesized nanoparticles and can be calculated the size average of particles, in present study the analysis images showed most nanoparticles with irregular cubic shape, it seem to be in mono-dispersed distribution .

Fourier-transform Infrared Spectroscopy (FTIR)

FTRI analysis offers qualitative and quantitative identify for organic and inorganic compounds in the samples as well identifies chemical pound in molecules through producing spectrum from an infrared absorption, the spectra which is produce represented profile for sample, FTIR analysis also provide information of the basis chemical composition and physical state of the whole samples, the unknown material are identified by searching the spectrum against a database of reference spectra.

Atomic Force Microscopy (AFM) analysis

AFM analysis was used to provided unique insight into the structure and functional behavior of synthesis IONPs in 3D and 2D images and have a much higher spatial resolution which offer the ability to investigated ultrafine structure of samples and even map the distribution of single molecule, the images was confirmed the surface topography, morphology and approximately size of synthesized iron oxide NPs.

E. coli Collection

Uropathogenic E. coli (UPEC) is common Uropathogens related in (UTIs), isolation and identification of bacteria through urine samples which was labeled, 5 ml from each urine samples were centrifuged at 3000 rpm for 5 min , the supernatant discharged and residue examined with light microscope by high power objected lines (40x), the samples containing 10 or more pus cells in one microscopic field are isolated as positive result, in same time the samples was cultured in different agar medium at 37 ^oC for 24 hr. the positive result recorded when 50-200 pure colonies growth in plat culture.

Evaluation of Minimum Inhibitory Concentration (MIC) of Fe_3O_4 NPs against E. coli

Macro-dilutions method was used to determine the (MIC) of Iron Oxide NPs required to inhibit the growth of E. coli bacteria, the method was carried out according to method recommended in[44] with some modification. capped tubes containing 2 ml of Muller-Hinton broth medium inoculated with 0.1 ml of $1.5x10^8$ cfu/ml of E. coli suspension prepared from overnight isolates cultured in nutrient agar, then 0.2 ml from each concentration 0, 50, 100, 150, 200, 250 µg/ml, was added and mixed to each 2ml MH broth of bacteria growth tube, after incubation at 37 ^oC for 24 h visual bacterial growth in tubes were observed and measured the optical density with spectrophotometer at 600 nm wavelength ,the high reading recorded to control tubes bacteria without NPs and the MIC was recording to the tube with the lowest concentration of IONPs without of visible growth of E.coli.

Antibiofilm activity of IONP

Biofilm is important reason for giving resistance to E. coli bacteria against conventional antibiotics, to experimented the effect of Fe_3O_4 NPs against E. coli biofilm formation in two methods ,Tube method(TB) and Congo red agar (CRA) method. The tube method was used for estimate qualitative of bacterial biofilm formation describe by[36],and assessment of the inhibitory effect of biogenic Fe3O4 NPs against biofilm formation by E .coli , two sterilized tubes with 10 ml of brain heart infusion broth with 2% sucrose medium inoculated with isolates, the first tube without any addition, the second tube well be mixed with MIC Fe_3O_4 NPs suspension at(1:1v/v) at concentration 200µg/ml and incubated 24 h at 37 ^oC After incubation , tubes was decanted and washed with phosphate buffer solution (PBS) pH 7.2 and dried then stained

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with 1% crystal violet ,after discharge excess CV ,washed and drying in inverted position and observed the antibiofilm activity, CRA method, A sample qualitative method for detection of biofilm production was describe by [37] ,and when incubation with suitable concentration of NPs can be used in detection the ability of NPs to inhibition bacterial biofilm formation, the method based on subculture of E. coli on brain heart infusion agar supplemented with sucrose and congo red dye, Positive result was indicted by black colonies and the addled of nanoparticles in known concentration prevented the formation of biofilm.

Antibacterial activity of IONP

Antibacterial activity of iron oxide NPs against E . coli cultured on Muller Hinton agar plates medium with standardized cell suspension of 0.5 McFarland turbidity ($1.5x10^8$) for 24 hr.at 37 °C, the method agar well diffusion were used to detected the activity of Fe_3O_4 NPs in concentration of 100, 200, 300, 400 µg/ml and the result was recorded the high inhibitions zone at high concentration.

RESULT AND DISCUSSION

Supernatant of Bacillus coagulans was used in biosynthesis of Fe_3O_4 NPs , the selective of bacteria based on the biologically activity of this bacteria, it was favored in industrial many type of enzyme production, lactic acid fermenter, bacteriocin secreted as well as spore forming and these characteristics are important in the diagnostic of these bacteria probiotic gram positive in addition to that identification through biochemical tests and morphological characteristics[38],clear yellow supernatant of B. coagulans well be changing to black brown color were consciously adding of NaOH 1M in dropwise on magnetic stirrer at 35 °C to mixture reactive of the iron salt precursor and the bacteria supernatant which was become more dark with time that indicting of IO nanoparticle production[39],the separation and collection of synthesized IONPs by used external magnet,(Fig. 1) and that confirm the magnetic nature of the nanoparticles.

FTIR spectra analysis of IONPs synthesis using bacteria B. coagulans was carried out to identify of chemical composition and functional group which is possible reduction of Fe ions or possible interaction between functional group and NPs hence could be act as capping agent helping in stabilization of nanoparticles. FTIR shown in (Fig. 2) absorption peaks at 574 cm^{-1} corresponding to Fe_3O_4 vibration related to magnetic phase [40] and Band at 3448 $cm^{-1}$is assigned to O-H stretch vibration of alcohols and band shown 2017 cm^{-1}, 1647 $cm^{-1}$are due to C-O stretching and 1346 cm^{-1} is attribute C=O the effective functional group of biomolecules in bacterial supernatant which could be carried out the interaction with iron salt precursors lead to biogenic of iron oxide NPs.

X-Ray diffraction analyses was used to characterized the dry powders of IONPs Fig. 3, from Debye-Scherer's equation the calculation of the average crystalline mean size of IONPs was (15,13 nm), The XRD diffraction of IONPs expressed peaks pattern at 2θ 31.50, 37.70, 45.20 analogous 220, 311, 440 crystal face of Fe_3O_4 of irregular cubic structure ,the positions and relative intensities of the reflection peak of iron oxide NPs agree with the X-ray diffraction peaks of standard

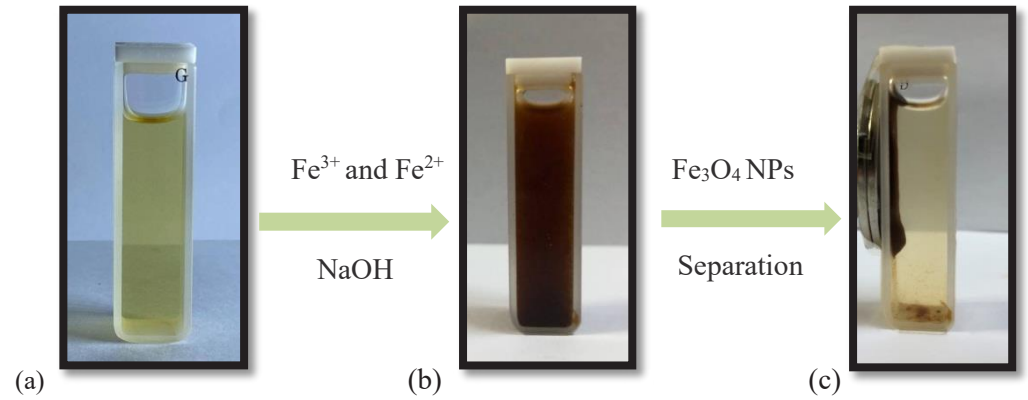

Fig. 1. (a) supernatant of *B. coagulans* (b)reactants (c) separation of Fe_3O_4 NPs from the mixture reaction solution by external magnet .

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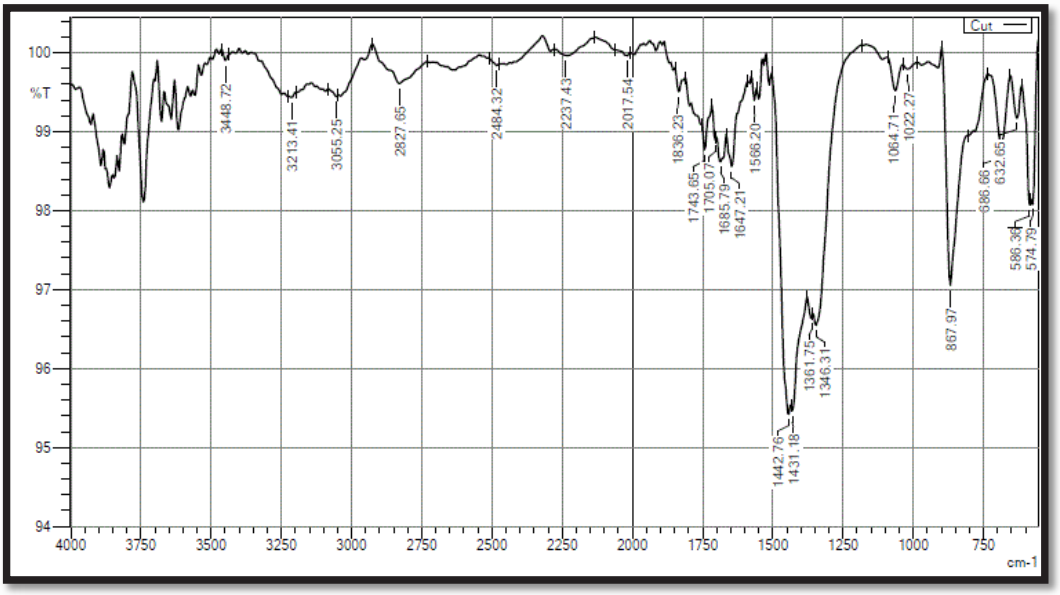

Fig. 2. FTIR spectrum of Fe_3O_4 Iron Oxide Nanoparticles.

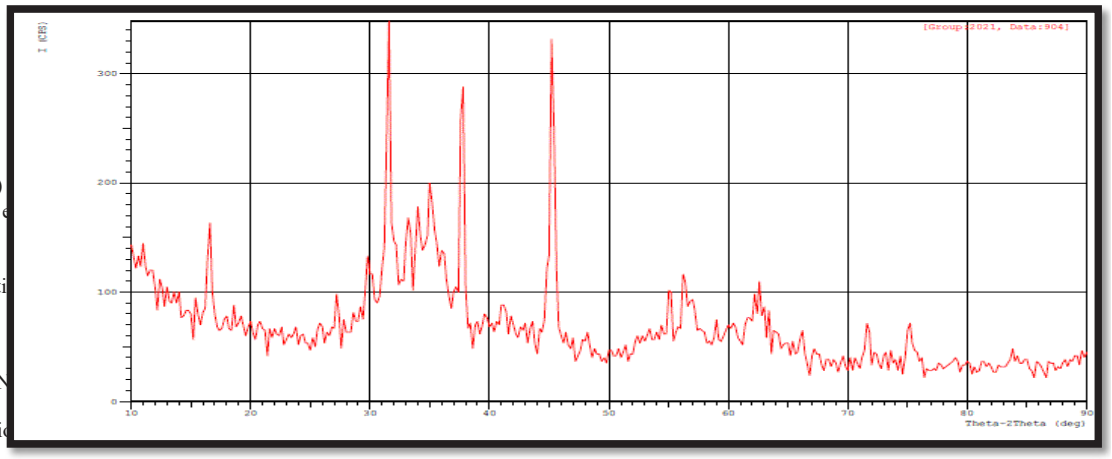

Fig. 3. X-ray diffraction of IONPs

Fe_3O_4 NPs samples and sharp peaks also suggest that the IONPs have good crystallize structure[41].

The SEM analysis confirm the information about the morphology and size of Nanoparticles, SEM Nanoscale images (Fig .4) of biosynthesis Fe_3O_4 NPs were appear in irregular cubic shapes and the average size of NPs (28.1) nanometers which is within the scale-range size of nanoparticles , the small size and irregular shape of this NPS were seen probably due to low level of agglomeration as result of fast formation of precipitation and short time of reaction incubation , furthermore abundance of active biomolecules and capping agent secreted by bacteria in growth medium when supernatant collection ,that could be considered protected agent by covered surface area of NPs which increased physical stability [42].

The AFM analysis give information about the topography and morphology of nanoparticles. IONPs were synthesized by bacteria supernatant was analysis with this technique to indicated the roughness and average diameter as well as to provided two-dimensional (2D) and three-dimensional (3D) images, Fig. 5.a,b. the images of section sample of the surface over a 1x1µm scan of the Fe_3O_4 NPs showed uniform height distribution

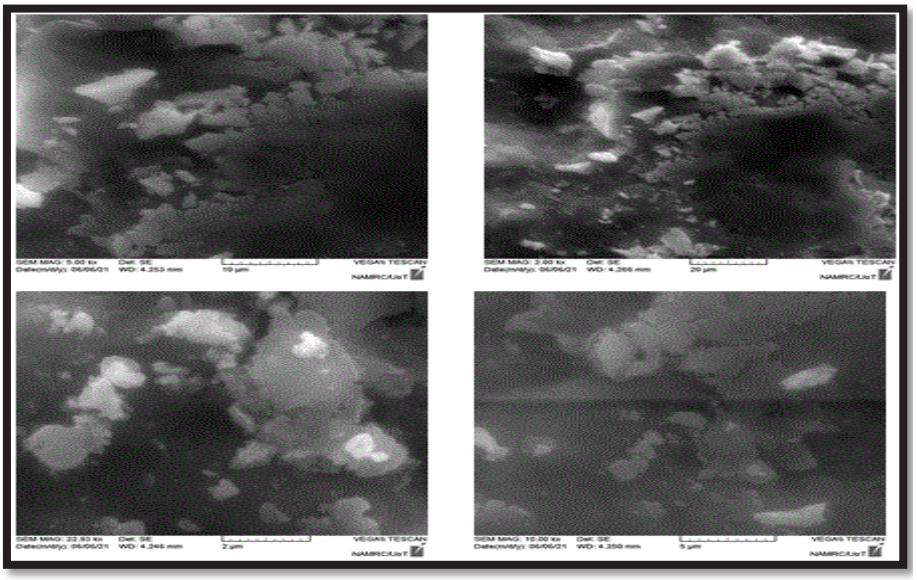

Fig. 4. Scanning electron microscope (SEM) of IONPs.

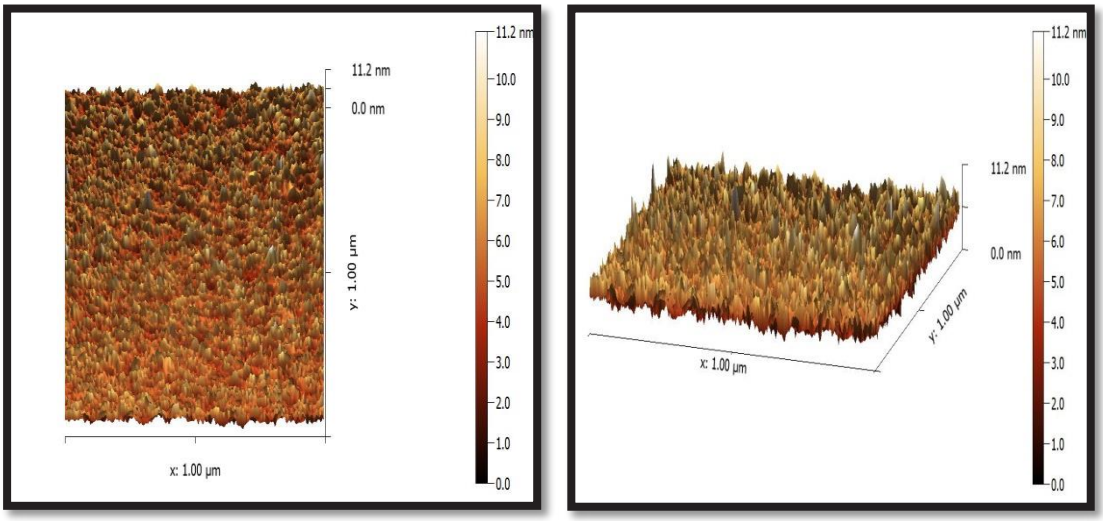

Fig. 5. AFM topography images. a: Two-dimensional b: Three-dimensional

around 11 nm, and the size of monodispersed single iron oxide NPs was 15,3 nm, and the crystal appear to tendency to form aggregations may be due to the attractive interaction between the nanoparticles, the particles size distribution in nanoscale and the means averages at 22,7 nm, the shape of nanoparticles look like irregular cubic were sharp angle stretch up formed a homogeneous surface appearance in which the upper part is spiky [43].

The minimum inhibitory concentration (MIC) assay was applied for assessment minimum bacteriostatic of the biosynthesized Fe_3O_4 NPs against E .coli bacteria in macro-dilution method. At concentration of 0, 50, 100, 150, 200, 250, 300 µg/ml of NPs with growth culture of tested bacteria ,the MIC values was at 150 µg/ml according to optical density (OD) in the spectrophotometer at 600 wavelength were the control growth tube without IONPs (0 tube) was recorded high OD value were compared with bacteria growth culture with NPs, the lowest concentration 150 µg/ml clearly inhibition cells number at lowest OD absorption.

Congo red agar and tube method used to

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evaluated the activity of Fe_3O_4 nanoparticles at concentration 150 µg/ml against biofilm formation of E. coli bacteria , the result showed in two method the iron oxide NPs can be reduction of biofilm formation by E. coli . the reduced of cell numbers and inhibited bacteria attachment to surfaces play an important role in formation of biofilm [45] small size and magnetic behavior of IONPs make possible to effective as antimicrobial and easy to penetration of biofilm matrix. In addition to high surface to volume ratio that facilitated the reaction and contact with bacterial cell wall of plankton that prevent attachment to aggregation or damage the DNA or may alter the gene expression relating to biofilm formation [46].

The antimicrobial activity of IONPs was investigated against Uropathogenic E. coli most common urinary tract infection(UTIs) pathogen by well diffusion method, four concentration of iron oxide NPs was used 100, 200, 300 and 400 µg/ml on Muller-Hinton agar medium ,the result showed that Fe3O4 NPs has antibacterial activity against E. coli bacteria in dose depended manner that mean the highest inhibition was observed at 400 µg/ml with respect to low concentration [47] ,the antibacterial activity of IONPs still unknown. However, the NPs involve in generation of reactive oxygen species (ROS) resulting in cell wall and bacterial membrane permeability disruption leading to cell death.

CONCLUSION

Free cells supernatant of B. coagulans was mediated synthesis of iron oxide nanoparticles as green stabilizer agent in co-precipitation method associated with biomolecules and metabolites in supernatant which are involvement in fast production of good crystallinity and small average size of the NPs, that based on different characterization analyses. The IONPs exhibit potential antimicrobial against E. coli in depend on concentration that efficacy rate increased when concentration increased and about the effectiveness of NPs against antibiofilm formation ,the MIC at 150µg/ml is sufficient to inhibition of biofilm formation that clearly showed in Congo red agar and tube method.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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