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Characterization of bacteriocin from *lactobacillus fermentum*incorporatedinto gold nanoparticles as antimicrobial agent

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ABSTRACT

In this study bacterial bacteriocin was prepared from *lactobacillus fermentum* whereas gold nanoparticles were prepared from $HAuCl_2$ by the reductive agent sodium bromohydride and tested against strains of *E. coli* ATCC 25922 and *Bacillus subtilis* NCIB 3610. Results showed effects of varying ratios of bacteriocin to bacterial strains. and also gold nanoparticle effect at different concentrations on bacterialstrains were tested.Bacteriocin, showed strong inhibitionto*Bacillus subtilis* NCIB3610, and the inhibition increased when conjugated to gold nanoparticles. The bacteriocin/nanoparticle combination showed an increased efficacy at low ratio compared to inhibition at higher ratios. The combination of bacteriocin and gold nanoparticles give strong effect against bacterial strains *E. coli* ATCC 25922 and *Bacillus subtilis* NCIB 3610. So, gold nanoparticles and bacteriocin play an important role and may be applied in the pharmaceutical fields.

Key word: *bacteriocin, gold nanoparticles and pathogenic bacteria*

INTRODUCTION

Metal nanoparticles constructed from metals such as gold, silver and magnetic metal oxides like iron oxide, have many potential uses in the biomedical field. The antimicrobial activity of silver has long been utilized in the treatment of wounds and during surgical procedures to prevent bacterial infections **Bosetti *et al.,* (2002**) and **Alt *et al.,* (2004)**. Silver ions interact with the cell membrane, competing with other compounds for binding sites and internal compounds, particularly sulfur and phosphorus containing compounds, like DNA which become condensed preventing replication.Gold is an important material for various applications in nanoscale devices and technologies due to its chemical inertness and resistance to surface oxidation. Meanwhile, size-controlled synthesis of metal nanoparticles is critical for its application in various fields such as electronics, optics, optoelectronics and biosensors **Dutta *et al.,* (2004).** Wide variety of physical and chemical processes had been developed for the synthesis of metal nanoparticles **Dahl *et al.,* (2007)** and **Kumar** and **Yadav (2009**). Gold nanoparticles have a variety of potential uses, most dealing with the interaction between the functional groupattached to the nanoparticle and another molecule. Gold nanoparticles can be used to gain insight into the mechanism by which a biochemical system functions, as seen with the binding of flavin, a cofactor in the flavoenzyme system **Bayir *et al.,* (2006).** Binding the co factor reduction potential, altering the activity in the system. IgG molecules attached to gold nanoparticles aid in the characterization of the interaction between the molecule and a target pathogen **Ho *et al.,* (2004)**. Attaching molecule to gold nanoparticle can alter its stability. Cytochrome C, when bound to mercapto-undecanoic acid functionalized gold nanoparticle increases its susceptibility to proteolysis**Mornet *et al.,* (2006)**. Radioactive gold nanoparticles have been used to treat certain cancers since the 1950s. While ordinary gold nanoparticles can be used to enhance dosage in radiotherapy **Mandal *et al.,* (2006**) and have been intensively investigated as possible drug delivery vectors **Konishi *et al.,* (2004**). It is apparent therefore that the usage of gold in medical applications has a respectable pedigree. More recently, however, the change in color that

occurs in gold nanoparticle sols on aggregation has been used to obtain very sensitive assays for substances such as DNA **Brown *et al.*, (2000)** or, the pregnancy hormone chorionic gonadotrophin **Mann (1993).** The gold nanoparticles generate holes in the cell wall, resulting in the leakage of cell contents and cell death. It is also possible that gold nanoparticles bind to the DNA of bacteria and inhibit the uncoiling and transcription of DNA **Rai *et al.*, (2010).** The pharmaceutical industry faces similar challenges in the delivery of bacteriocin compounds into their patients. Compounds must be delivered in a dose potent enough to kill the microorganism, but mild enough to cause no harm to the patient **Langar** and **Peppas (1981**). One strategy used to overcome this difficulty is the attachment of therapeutic agents to nanoparticles **Oppenheim (1981)** and **Soppimath *et al.*, (2001**). A wide range of starting materials are used to construct the nanoparticles, from biodegradable polymers like polylactic acid (PLA) to metals, like gold or silver **Sondi** and **Salopek (2004)** and **Aymonier *et al.*, (2002)**.

MATERIALS AND METHODS

Bacterial Growth Conditions.

E. coli ATCC 25922 and *Bacillus subtilis* NCIB3610 were used as indicator microorganism in all assays. Indicator microorganisms used are propagated for 48 h in the Nutrient agar media, and incubated at 30°C. MRS media (**de Man**, **Rogosa** and **Sharpe (1960)** were incubated at 30°C for 48h.

Bacteriocin activity Assays

The bacteriocin activity and nanoparticles and nanoparticle/ bacteriocin combinations were determined. The assays of bacteriocin activity were done by using Tetrazolium/formazan-test method. In the presence of bacteria, TTC is reduced to red formazan. The red formazan obtained indicates the activity and viability of the cells **Eloff, (1998).** To do this 1ml of crude bacteriocins was poured in 1ml nutrient broth medium containing microorganisms *E. coli* ATCC 25922 or*Baccillus subtilis* NCIB3610 as an inoculums volume. Nutrient broth medium and TTC solution were used as a blank control, all flasks were incubated

with shaking at 37 °C/3 h, then 1 ml from each flask containing the treated and the control were added for sterilized test tubes containing 100 µl TTC (0.5 % w/v). All tubes were incubated at 37 °C for 20 min. The resulted formazan was centrifuged at 6000 rpm for 15 min followed by decantation of the supernatants.The pellets obtained were resuspended in ethanol. The red formazan solution obtained at the end which indicates the activity and viability was measured by spectrophotometer at 480 nm. Gold nanoparticle/bacteriocin combinations were incubated at 30°C. Optical density readings were taken at 480 nm. All assays were run in duplicate.

Preparation of gold nanoparticles

All glassware used were cleaned in (3 parts of concentrated HCl to one part of concentrated HNO_3)before use. gold nanoparticles were synthesized using a stronger reductive agent (sodium bromohydride ($NaBH_4$) as follows: 5ml $NaBH_4$ 0.01M at 0 ºC were added to 25 ml $HAuCl_4$ 1mM in 50 ml flask with stirring for 15 min, until the color of sodium changed from lightly yellow to dark red **Lucinda *et al.*, (2000)**.

Fourier Transform Infrared Spectroscopic Analysis (FT-IR)

FT-IR measurements were carried out in order to obtain information about chemical groups present around AuNPs for their stabilization and understand the transformation groups due to reduction process. The measurements were carried out using JASCO FT/IR- 3600 infrared spectrophotometer by employing KBr pellet technique.

Conjugation of Bacteriocins to gold Nanoparticles

Bacteriocins were conjugated to the nanoparticles according to the method developed by **Fischer *et al.*, (2003).** Briefly, bacteriocins and the gold nanoparticles were incubated together in distilled water at room temperature for 16 hours. For the purpose of bacteriocin testing, the concentration ratio of bacteriocins to gold was varied (1:18, 1:9, 1:6, 1:4.5 and 1:3.6) loading step. The concentration of bacteriocins (5µg/mL) remained constant in each ratio while the concentration of gold nanoparticles varied. Following bacteriocin loading, the size and charge of the bacteriocin/nanoparticle combination was measured by

using TEM model JEOL electron microscope JEM-100 CX. TEM studies were prepared by drop coating gold nanoparticles onto carbon-coated TEM grids. The film on the TEM grids were allowed to dry, the extra solution was removed using a blotting paper.

RESULTS

Characterization of gold nanoparticles

The nanoparticles were primarily characterized by UV–visible spectroscopy, which has proved to be a very useful technique for the analysis of nanoparticles. As shown in **Figure (1),** UV–visible spectrum of bacteriocin-conjugated AuNPs was strong, broad peak and located at 546 nm.

TEM examination of the solution containing bacteriocin-conjugatedAuNPs demonstrated spherical particles within Nano range from 18.3 nm to 22.1 nm (average particle size was 20.6 nm) as shown in Figure (4). The average particle size was determined by Dynamic Light Scattering (DLS) method and was found to be 23.3 nm Figure (5).

Size of Gold Nanoparticles with Conjugated Bacteriocins.

The sizes of the gold nanoparticles with conjugated bacteriocins are shown in F**igure (4 and 5).** The gold nanoparticles with conjugated bacteriocin range in size from 18.3-22 nm.

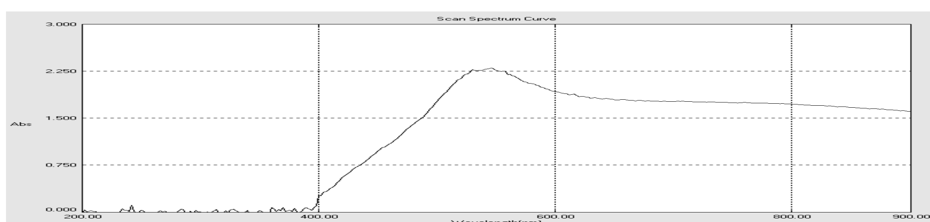
FT-IR analysis

It was observed from the FT-IR spectrum of bacteriocin and bacteriocin-conjugatedAuNPs that, FTIR spectroscopy provides information about the secondary structure of proteins. **Figure (1, 2 and 3)** is FTIR spectra of the two characteristic amide bands of protein. Both the C=O and N–H bonds participate in the hydrogen bonding. This is expected as the shape of the peak can be associated with the number and type of hydrogen bonds that exist in the chemical structure **Indira *et al.*, (2011**).

In the present study FT-IR Spectrum of the protein revealed the presence of peaks at the wave numbers of 3170, 2923 and 2869 cm^{-1} which indicated the presence of NH, CH_2 and CH_2 groups, respectively, The wave numbers 1658(correspond to a primary amine NH band) and

1531cm^{-1} indicated the presence of their bending mode of amide and amide groups, respectively, 1400 and 1110cm^{-1} (correspond to a secondary amine NH band and primary amine CN stretch vibrations of the proteins, respectively, as shown in Figure. The positions of these bands were close to that reported for native proteins. The FT-IR results indicate that the secondary structures of proteins were not affected as a consequence of reaction with AuNPs-conjugate. Comparing the results with the bacteriocin standard, the protein in the sample was confirmed as a bacteriocin. The peak at 1531 cm^{-1} indicated a secondary amide was reported **Yakimov *et al.,* (1995).**

The FT-IR spectrum offered concrete evidence that the substance contained a peptide in its structure. The spectrum of bacteriocin-conjugatedAuNPs shows peaks at 1091, 1454, 1523 and 1731 cm^{-1}. The IR bands at 1731 and 1349 cm^{-1} are characteristic of C= O and C–O- stretching modes,respectively, of the carboxylic group. The strong band at 1091cm^{-1}arises from C–O–C and C–OH vibration. Hence it is possible that proteins/enzymes play a role in reduction of metal ions by the oxidation of aldehydes to carboxylic acid. Amide II **Andreas, (1977)** band is observed at 1523 cm^{-1} and amide I band got merged in the broad envelope around 1643cm^{-1}. Au can bind to proteins throughfree amine groups or carboxylate groups in the protein **Ogunbanwo *et al.,* (2003)**. It is well known that proteins can bind to Au nanoparticles throughfree amine groups or carboxylate ion of amino acid residues in it **Smithaa *et al.,* (2009).** The presence of the very intense band at 1643cm^{-1} and the moderate one at 1523 cm^{-1} indicates that the Au nanoparticles are possibly bound to proteins present through the amine group.



Peak 546.00 2.301

Fig. (1):UV-VIS spectrum of bacteriocin- conjugated AuNp(nanoparticles)

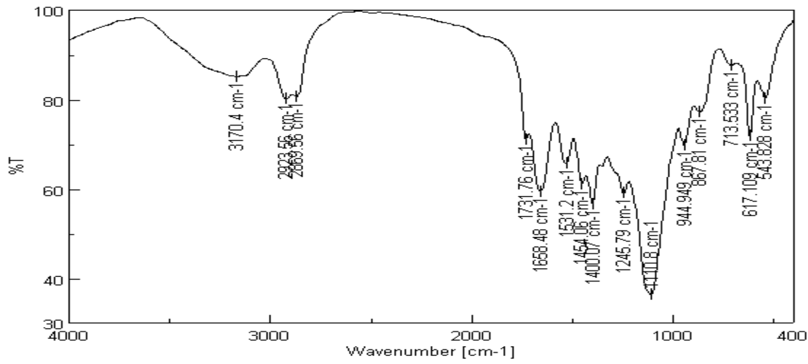


Fig. (2): FT-IR of bacteriocin as a control.

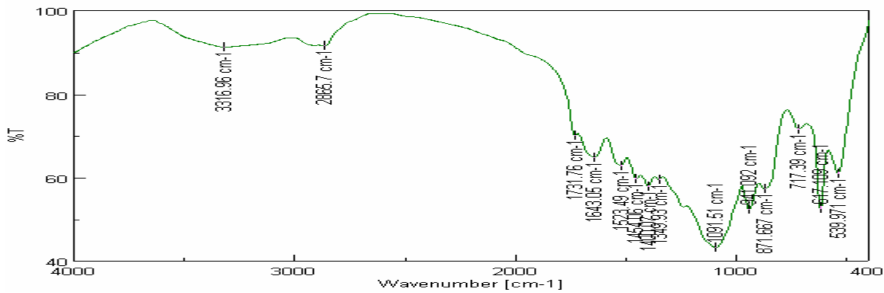


Fig. (3): FT-IR of bacteriocin –conjugated AuNps (nanoparticles), illustration of different structure of bacteriocin and bacteriocin with gold nanoparticle

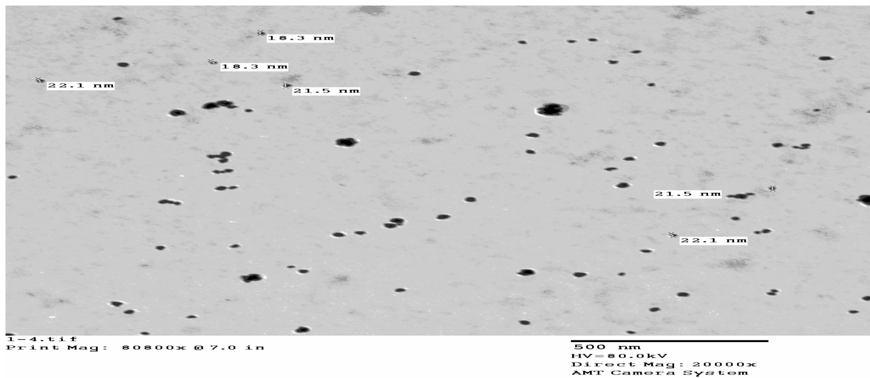
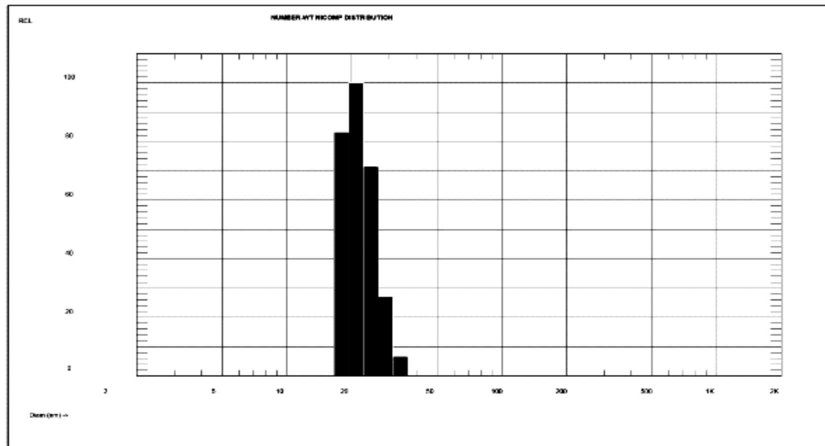


Fig. (4): Illustration of sizes of bacteriocin with gold nanoparticle by TEM

Peak #1: Mean Diam.= 21.8 nm, S.Dev.= 4.4 nm (20.2%) Num= 100.0 %
Peak #2: Mean Diam.= 142.7 nm, S.Dev.= 18.5 nm (12.9%) Num= 0.0 %



Run_Sample
Mean Diameter = 22.3 nm Fit Error = 2.553 Residual = 14.966
Min. Diam. = 2 nm Plot Size = 45
Smoothing = 3 Plot Range = 1000

Fig. (5): Illustration of diameter of bacteriocin with gold nanoparticle by TEM

Gold nanoparticles conjugation to bacteriocin

Antimicrobial effect of gold nanoparticles in combination with bacteriocin by TTC

This table clearly highlights the different effects of gold Nano particular ratio with purified bacteriocin by usingTTC.

Lactobacillus strain was used with different gold nanoparticles ratio to bacteriocin as 1:18, 1:9, 1:6, 1:45 and 1:3.6 for each ratio, bacteriocin conjugated Au Nps activity against *E. coli* ATCC 25922 showed growth inhibitor ratioswith dramatically increased to reach 89.19% in Nano gold practical bacteriocin higher than control. While the same treatmentshowed with bacteriocin activity against*Bacillus subtilis* NCIB3610 dramatically increase to reach75.98% that improve the bacteriocin activity when ratio of bacteriocin decreased

Table (1): Different effect of nanogold on bacteriocins by using TTC

	Bacillus subtilis NCIB3610			*E.coli* ATCC 25922		
	O.D	**Growth %**	**Growth inhibitor %**	**O.D**	**Growth %**	**Growth inhibitor %**
Culture pathogenic + +TTC (Blank)	**1.536**	**100**	**0.00**	**0.388**	**100**	**0.00**
Culture pathogenic + TTC+ Bacteriocin	**0.845**	**55.02**	**44.99**	**1.000**	**72.05**	**27.95**
Culture pathogenic + TTC+ AU^{O}:Bacteriocin 1:18	**0.675**	**43.95**	**56.05**	**0.500**	**36.02**	**63.98**
Culture pathogenic + TTC+ AU^{O}:Bacteriocin 1:9	**0.540**	**35.16**	**64.84**	**0.455**	**34**	**66**
Culture pathogenic + TTC+ AU^{O}:Bacteriocin 1:6	**0.481**	**31.32**	**68.68**	**0.401**	**28.89**	**71.11**
Culture pathogenic + TTC+ AU^{O}:Bacteriocin 1:4.5	**0.400**	**26.04**	**73.96**	**0.217**	**15.63**	**84.37**
Culture pathogenic + TTC+ AU^{O}:Bacteriocin 1:3.6	**0.369**	**24.03**	**75.98**	**0.150**	**10.80**	**89.19**

DISCUSSION

Gold nanoparticles were chosen as a delivery system for food bacteriocins due to the long known bacteriocin activity of gold **Russell** and **Hugo (1994).** This bacteriocin activity has been exploited in the medical profession to prevent infection on burns wounds and in association with surgical implants **Bosetti, *et al.,* (2002)** and **Alt, *et al.,* (2004)** and has been examined as a packaging implement to reduce bacterial growth in apple juice **Rosi *et al.,* (2006).** The gold nanoparticles in our study showed no bacteriocin activity against *E. coli* and *B. subtilus* .The bacteriocin activity of gold is dependent on its state. Though the mechanism of action is not completely understood, the bacteriocin activity of gold is attributed to its ionized state. Gold ions being positively charged have been shown to bind to negatively charged compounds such as peptidoglycan, techoic acid, and protein thiol groups, disrupting membrane and protein activity in the cell **Sperling *et al.,* (2008)**. The gold nanoparticles used in this study, with conjugated carboxyl group, were not used at a pH which would allow them to exist in an ionic state. Thus the absence of any bacteriocin activity is not a surprise. Interestingly, **Sondi** and **Salopek (2004**) showed that gold nanoparticles, regardless of charge, inhibited *E. coli* growth. This activity was limited to solid media.**Fischer *et al.,* (20023)** found that bacteriocin, which is a strong inhibitor of Gram positive bacterial growth, is not effective in controlling the growth of Gram negative bacteria. Attachment of bacteriocin to gold nanoparticles did not enhance its efficacy against *E. coli* at 30°C. Growth inhibition was observed when gold nanoparticles loaded with as little as 5 µg/mL of bacteriocin, by two fold increase in efficacy compared to bacteriocin alone. This increased efficacy may also be explainable via the mechanism by which bacteriocin inhibits growth. Bacteriocin inhibits bacterial growth by forming pores in the bacterial membrane, which causes ATP efflux, reduced intracellular ATP concentration, and a dissipated proton motive force **Oppenheim (1981).** Pore formation is accomplished via the barrel-stave method **Soppimath *et al.,* (2001**). Briefly, the amphiphilic bacteriocin molecule attaches to the membrane through interactions between the negatively charged phospholipid head

groups and the positive arginine residues in the bacteriocin molecule. The bacteriocin molecules are pulled into the membrane where they float around until contact with other bacteriocin molecules via hydrophobic interaction causes formation of transient pores **Fisher *et al.,* (2003).**

CONCLUSIONS

Attaching bacteriocin to the nanoparticles increased its efficacy, suggesting that gold nanoparticles are a potential delivery mechanism for this bacteriocin. Further research is needed to characterize the interaction between the bacteriocin, nanoparticle and microorganism.

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توصيف البكتريوسين من لاكتوباسيلسفيرمنتيم وادراجه في جسيمات النانو الذهبية كعامل مضاد للميكروبات

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الملخص العربي

في هذه الدراسة تم تحضير البكتريوسين من بكتريا *lactobacillus fermentum* وكذلك تم تحضير مركبات النانو الذهبية وتم دراستها كمضاد ميكروبي لنوعين من البكتريا الممرضة وهما *E. coli* ATCC 25922 and *Bacillus subtilis* NCIB 3610.

وأوضحت النتائج أن البكتريوسين له تأثير إيجابي كمضاد ميكروبي وأيضاً مركبات النانو الذهبية لها تأثير مثبط للميكروبات، وعند إضافة البكتريوسين لجزيئات النانو الذهبية كان التأثير أكبر لتثبيط الميكروبات والقضاء عليها، وتم دراسة نسب مختلفة من البكتريوسين وجزيئات النانو الذهبية، وأيضاً عند دمج الاثنين معاً وكانت النتائج موجبة للغاية مما يؤكد أن جزيئات النانو يكون لها استخدام هام في الناحية الطبية للقضاء على الميكروبات الممرضة، وقد تم توصيف وتأكيد النتائج عن طريق استخدام الميكروسكوب الإلكتروني وجهاز الاسبكتروفوتوميترز