EFFECT OF TITANIUM NANOPARTICLES BIOSYNTHESIS BY *LACTOBACILLUS CRISPATUS* ON UREASE,HEMOLYSIN& BIOFILM FORMING BY SOME BACTERIA CAUSING RECURRENT UTI IN IRAQI WOMEN

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Abstract

The development of reliable and eco-friendly biosynthetic process of titanium nanoparticles acts as a vital role in the field of nano-biotechnology today. The present investigation reported that extracellular rapid biosynthesis of titanium nanoparticles using *Lactobacillus crispatus* isolated from heathy women vagina. The characterization of nanoparticles were carried out by atomic force Microscopy (AFM). AFM analysis was confirmed that nanoparticles size in range of (70.98)nm. The potential of nanoparticles to control the formation of biofilms, as a function of their biocidal, anti-adhesive, and delivery capabilities, is now coming under close scrutiny.biologically synthesized titanium nanoparticles played a potential role in significant higher antimicrobial efficacy against multi drug resistant caused recurrent urinary tract infection *Klebsiella pneumonia, Staphylococcus aureus Acinetobacter baumani.,E.coli&Morganella morganii* and its virulence factors(biofilm formation,hemolsin& urease) were found asignificant effects after treated those isolates with subMIC (16mg/ml) titanium nanoparticle together and cultured on their special media .Our results showed that TI-NP had inhibitory effect against recurrent UTI causative bacteria at concentration (32) mg\ml &TI nanoparticles with appropriate concentration (16)mg/ml could show significant reduction in biofilm formation.hemolysin &urease production..

Keywords: Titanium nanoparticles, Iraqi women

Introduction

Urinary tract infection (UTI) is a serious health problem affecting millions of people each year. It is estimated that there are about 150 million cases in the world per year (Stamm & Norrby, 2001). The recurrence rate is high and often the infections tend to become chronic with many episodes .Many bacterial infections are associated with biofilm formation. In the urinary tract bacterial biofilms develop on both living surfaces and artificial implants, producing chronic and often intractable infections. Biofilms are defined as microbially derived sessile communities characterized by the cells that are irreversibly attached to a substratum or to each other. They are embedded in a matrix of extracellular polymeric substances (EPS) they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription.(Donlan *et al*.,2002). Microorganisms growing in a biofilm are intrinsically more resistant to antimicrobial agents than planktonic cells. High antimicrobial concentrations are required to inactivate organisms growing in a biofilm, as antibiotic resistance can increase 1,000 fold.(Stewart *et al*.,2001), Both Gram-positive and Gram-negative bacteria have the capability to form biofilms. Bacteria commonly involved include *Enterococcus faecalis, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus viridans, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis* and *Pseudomonas aeruginosa*.(Donlan et al.,2001).

Recent studies on the use of microorganisms in the synthesis of nanoparticles are relatively new and exciting area of research with considerable potential for development. The recent discovery of biosynthesis of metal nanoparticles points towards new biotechnological methods in materials science .The non-toxic and biocompatible properties of titanium find its applications in biomedical sciences such as bone tissue engineering as well as in pharmaceutical industries(Jayaseelan.etal.,2013)

2-Materials and Methods

2.1 microorganism

-Isolate of *Lactobacillus crispatus was isolated from* vagina of Iraqi healthy women, then identified through out cultural , microscopical and biochemical test according to (Kandler and Weiss,1986;Hammes and Vogal,1995 ;Carr *et al*.,2002). Five isolates were used to test the antibacterial , antiadhesive properties of the TI-NPS produced by *Lactobacillus Crispatus*, including *Escherichia coli , Klebsiella pneumonia , Morganella morganii ,Acinetobacter baumani* and *Staphylococcus aureus* .These isolates were isolated from urine samples of Iraqi women suffering from recurrent Urinary tract infection, then identified through out cultural , microscopical , biochemical test according to the criteria established by (Forbes *et al* ., 2002) and Vitek 2 system. Morover , these isolates were

tested for susceptibility to antibiotics, urease production & biofilm formation.

2.2 Detection for TINPs production

Three tubes will used for each isolates and each tube were filled with 40 ml of MRS broth solution . Then 20 ml of TiO2(0.025m) were added to the first and second tube respectively and both were shacked for half hour by magnetite steerer while the third tube contain MRS Broth only. Final concentration ultimately would be equivalent. Each isolates will cultured in first and third tube into CO2-incubator under temperature of 37C^{o} degree for 24,48,72 hours. Second tube was used as blank for first one , the change in color from light brown to dark brown observed and production of sediment will observed as primary detection of produced TINPs(Azhar *et al*.,2011).

2.3 Characterization of titanium nanoparticles by Atomic Force Microscopy:

Atomic Force Microscopy image was taken using Park system AFM XE 100. The aqueous titanium nanoparticles were deposited onto a freshly cleaved mica substrate. The sample aliquot was left for 1 min and then washed with deionized water and left to dry for15 min. The images were obtained by scanning the mica in air innon – contact mode (Daniel *et al.*,2012). The size,shape, and dispersity mode of TINPs will characterized by Atomic Force Microscopy .

2.4 Antibacterial activity

Antibacterial activity of TI-NPS were determined on the basis of minimum inhibitory concentration (MIC) values, defined as the lowest concentration of biosurfactants at which no visible growth could be observed after incubation for the required time. MIC was determined for *E. coli , K. pneumonia , Morganella morganii ,Acinetobacter baumani.* and *S. aureus* by Broth dilution method as described by Morello *et al.*,(2003). Briefly, a stock solution of TI-NPS from *L.crispatus* in sterilized distalled water were diluted to concentrations ranging (4,,8,16,32,64,128) mg/ml.

5.Effect of TI- nanoparticle on bacterial biofilm using Congo Red Agar Method

Slime production by 5 clinical isolates(*Klebsiella pneumonia, Staphylococcus aureus Acinetobacter spp.,E.coli&Morganella morganii)* were studied by congo red agar method. Briefly, Brain heart infusion agar supplemented with 5% sucrose and Congo red (0.08 g/l) was prepared. Congo red was prepared as a concentrated aqueous solution and autoclaved at 121°C for 15 minutes separately from other media constituents and was

then added when the agar had cooled to 55°C. (I ml)Ti-NP(subMIC) poured on congo red medium,left in room temperature to dry completely then Plates were inoculated and incubated aerobically for 24 to 48 hours at $37C^0$. Biofilm production was indicated by black colonies with a dry crystalline consistency whereas biofilm non-producers remain pink, though occasional darkening at the center of the colony was observed. A darkening of colonies with absence of crystalline colony morphology indicates an indeterminate biofilm production(Freeman,*et al.*,1989,Blanco.,*et al.*,2005)

2.6 Effect of TI- NPS on bacterial biofilm using Tube method (TM)

A qualitative assessment of bifilm formation was determined as previously described by(Christensen *et al*.1982). BHI+2% sucrose (0.5mL)+0.5ml sub MIC TI-NP(16mg/ml) was inoculated with loopful of microorganism from overnight culture plates .in the other hand 1ml BHI/sucrose was inoculated with loopful of test bacteria(as acontroll) and incubated for 24 hours at 37°C. The tubes were decanted and washed with distilled water and dried.dried tubes were stained with crystal violet (0.1%). Excess stain was removed and tubes were washed with deionized water. Tubes were than dried in inverted position and observed for biofilm formation. Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. Ring formation at the liquid interface was not indicative of biofilm formation.

2.7 Anti-adhesive activity of TI-NPS using tissue culture plate method (TCM)

The anti adhesive activity of TI-NP (subMIC 16mg/ml) studied against most multi resistant antibiotic bacterial isolates(5 isolates) under this study using microtitration plate methods(Mathur *et al*.,2006) performed in co- incubation experiments. (100 µl) Bacterial suspension in brain heart infusion broth with 2% sucrose were added with (100 µl) subMIC TI-NP together in the same well for each isolate,as acontrol. next well filled with(180 µl) brain heart infusion broth with 2%sucrose& (20 µl) bacterial growth suspension .plate was incubated at $37C^0$ / 24hour. non adherent cells will removed by gently washing twice the wells with steril distilled water plates let to dry in room temperature then stained with 200 µl crystal violet solution for 20 min,excess stain was rinsed off by through washing with deionized water & plates were kept for drying for 15 min at room temperature then 200 µl ethanol (95%) added for each well & read using microElisa autoreader.biofilm development was assessed by measuring the optical density (absorbance at 630nm) using a spectrophotometer. Data are presented as percentage change in biofilm growth in the presence of

nanoparticles with respect to absence of nanoparticles (control).Optical density OD stained adherent bacteria determined by

% Inhibition of adhesion = [1- ($\frac{A}{Ao}$)× 100]

A represents the absorbance of the well with a TI-NPS and A_0 the absorbance of the control well. The microtitre-plate antiadhesion assay estimates the percentage of bacterial adhesion reduction in relation to the control wells, which were set at 0% to indicate the absence of TI-NPS and therefore of its anti-adhesion properties. In contrast, negative percentage results indicate the percentage increase in microbial adhesion at a TI-NPS given in relation to the control.

2.8 Effect of TI- NPS on bacterial urease production

urease production by 5 clinical isolates(Klebsiella pneumonia, Staphylococcus aureus, Acinetobacter baumanis.,E.coli&Morganella morganii) were studied by urease agar method according to Malarkodi *et al*.,(2013) with some modification. Briefly. (I ml)Ti-NP(subMIC) poured on urease agar medium,left in room temperature to dry completely then Plates were inoculated and incubated aerobically for 24 hour at $37C^0$. urease production was indicated by pink colonies where as urease non-producers remain yellow.

2.9 Effect of TI- NPS on bacterial hemolysin production

hemolysin production by 5 clinical isolates(Klebsiella pneumonia, Staphylococcus aureus, Acinetobacter baumanis.,E.coli&Morganella morganii) were studied by blood agar method according to Malarkodi *et al*.,(2013) with some modification. Briefly. (I ml)Ti-NP(subMIC) poured on blood agar medium,left in room temperature to dry completely then Plates were inoculated and incubated aerobically for 24 hour at $37C^0$.

3-Results and Discussion

The pure culture of *Lactobacillus crispatus* was inoculated in MRS broth, Morphological examination of all *lactobacillus* isolates grown on MRS agar medium showed white, large, smooth, round colonies with entire margin. In microscopic examination, the cells of *Lactobacillus* reacted positively with Gram stain, rods with rounded ends that occurred singly, in pairs or in short chains and non-spore forming. All bacterial isolates were catalase, oxidase and gelatinase negative. (Kandler and Weiss,1986 ; Carr *et al*., 2002) .

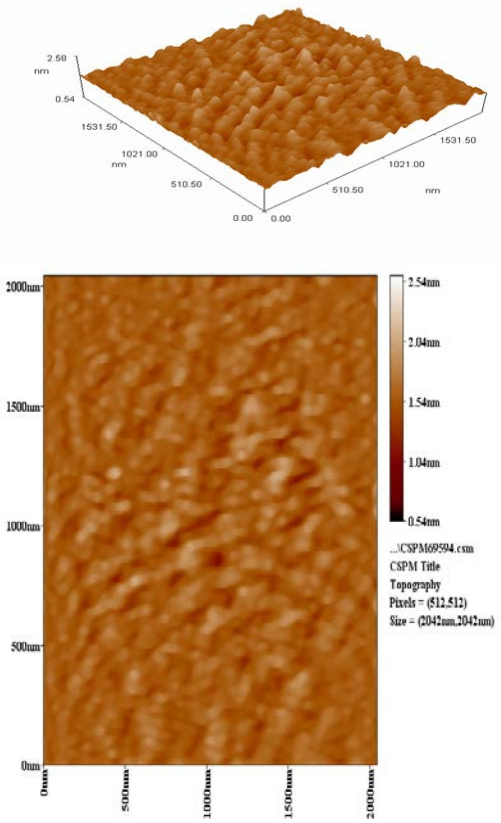
Biosynthesis is the phenomena which takes place by means of biological processes or enzymatic reactions. These eco-friendly processes are referred as green and clean technology, and can be used for better synthesis

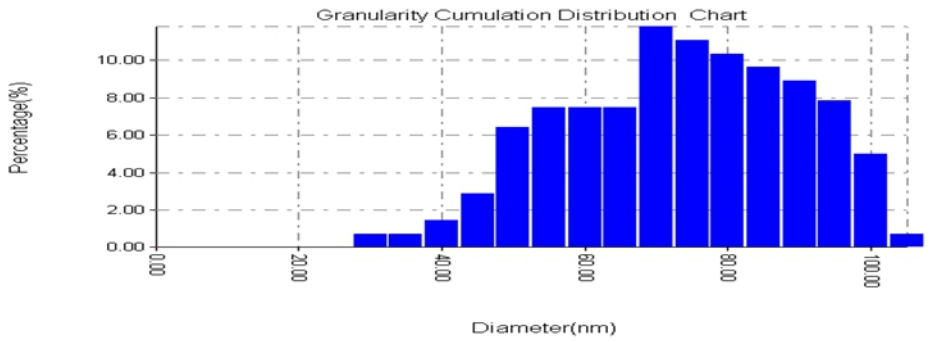
of metal nanoparticles from microbial cells. Microorganisms can survive and grow in high concentration of metal ion due to their ability to fight against stress(Moghaddam,2010). *Lactobacillus crispatus* isolate was tested for TINPs production. Titanium dioxide exposing to the bacteria was reduced and nanoparticles of titanium formed. Solution color changed from light brown to dark brown in first tube ,second tube used as controls shown in figure (1). According to Beveridge (1997), the mechanisms which are considered for the biosynthesis of nanoparticles includes efflux systems, alteration of solubility and toxicity via reduction or oxidation, bioabsorption, bioaccumulation, extracellular complexation or precipitation of metals, and lack of specific metal transport systems . Nair and Pradeep (2002) observed that during the initial step of synthesis of nanoparticles by lactobacillus, nucleation of clusters of metal ions takes place, and hence there is an electrostatic interaction between the bacterial cell and metal clusters which leads to the formation of nanoclusters, the smaller sized nanoclusters get diffused through the bacterial cell wall.

Figure(1): left tube *Lactobacillus crispatus* in MRS broth without TIO2 taken as control (B) right tube with bacteria and TIO2 (0.025 mM) while middle tube is blank TIO2 and MRS broth only.

Azhar. *et al*.,(2011) and Jha & Parasad (2010) found extracellular Lactobacillus-mediated Biosynthesis of Titanium Nanoparticles in MRS-broth Medium ccurred anaerobically after 72h with nanoparticle size 150nm from dairy products .while Azhar& Ladan(2011) offered novel method for synthesis of nanoparticles that contains high frequently potential in low cost nanomaterial production with echo friendly condition and high yield possibility.they used Titanium dioxide with *Lactobacillus plantarum* in controlled conditions and titanium nanoparticles was formed. AFM was used to view the nanoparticles both in surface and three Dimensional view ,and

found the average size of particle (70.98)nm as shown in figure(2). Among several oxide semiconductors, TiO2 has a more helpful role in our environmental purification due to its non-toxicity, photo catalytic activity, photo induced super-hydrophilicity and anti-fogging effect. These properties have been applied in removing bacteria and harmful organic materials from water and air, as well as in self-cleaning or self-sterilizing surfaces in medical centers TiO2 is widely used as an ingredient in white paint and building materials, food colorant, sunscreen and cosmetic products.The opacity and whitening properties of TiO2 NPs have a variety of industrial applications,





Figure(2): Atomic Force Microscopy image of titanium nanoparticles synthesized by *lactobacillus crispatus*

including the manufacture of paints,textiles, papers, plastics, sunscreens, cosmetics, and food products(Jayaseelan *et al.*,2013)

Biofilm producing bacteria are responsible for many recalcitrant infections and are notoriously difficult to eradicate. They exhibit resistance to antibiotics by various methods like restricted penetration of antibiotic into biofilms, decreased growth rate and expression of resistance genes. There are various methods for biofilm detection.(Christensen *et al.*,1995).

In this study we evaluated five isolates by three screening methods for their ability to form biofilms.(Mathur *et al*.,2006) in presence of titanium nanoparticles produced by *L .crispatus*.as showed in(table 1,fig3), the antiadhesive activity of TI-NP was evaluated against recurrent UTI causative bacteria. TI-NP showed antiadhesive activity against all bacteria except *E.coli*(-3) ,the highest antiadhesive percentage was observed for *Morganella morganii* (48%) .On the contrary, low activity was obtained for *Acinetobacter baumani* (6%) .

Microbial biofilms on biomaterial implants or devices are hard to eliminate by antibiotics due to their protection by exopolymeric substances that embed the organisms in a matrix, impenetrable for most antibiotics and immune-cells. Application of metals in their nanoparticulated form is currently considered to resolve bacterial infections.

Table 1: Antiadhesive activity of titanium NP isolated from *Lactobacillus crispatus*

Bacterial isolates	**(O.D) optical density**		**Antiadhesive Percentage(%)**
	Control	**Treatment**	
Staphylococcus aureus	0.068	0.060	12
Escherichia coli	0.067	0.069	-3
Acinetobacter baumani.	0.064	0.060	6
. klebsiella pneumonia	0.070	0.059	16
Morganella morganii	0.158	0.082	48

Positive percentages indicate the reductions in bacterial adhesion when compared to the control, and negative percentages indicate increased bacterial adhesion (-)Mean no inhibition on the other hand, The inhibition activity of TI-NP was evaluated on biofilm formation of UTI causative bacteria using tube method.TI-NP showed inhibitory effect on biofilm formation of all bacteria tested with different propotion except *E.coli* (Table 2)..Maurer-Jones *et al*.(,2013)observed, significant changes in bacterial growth biofilm growth, and riboflavin secretion of *Shewanella oneidensis* occurred after exposure to TiO2 nanoparticles. These changes were not the result of oxidative stress, but the proximity of the nanoparticles caused altered gene expression relating to biofilm formation and growth are the mxdABCD complex. Applerot *et al*.,(2011) evaluated the ability of glass slides coated with zinc oxide (ZnO) nanoparticles to restrict the biofilm formation of common bacterial pathogens. The generation of hydroxyl radicals, originating from the coated surface, was found to play a key role in antibiofilm activity&. antibacterial activity .

Table 2 :Effect of TI-NP on biofilm formation using tubes methods

Bacterial isolates	**Biofilm formation**	
	Control	**Treatment TI-NP**
S.aureus	+++	++
E.coli	++++	++++
Acinetobacter baumani	++++	++
K.pneumoniae	++++	++
Morganella morganii	+++	+

(+) weak, (++) moderate , (+++) strong

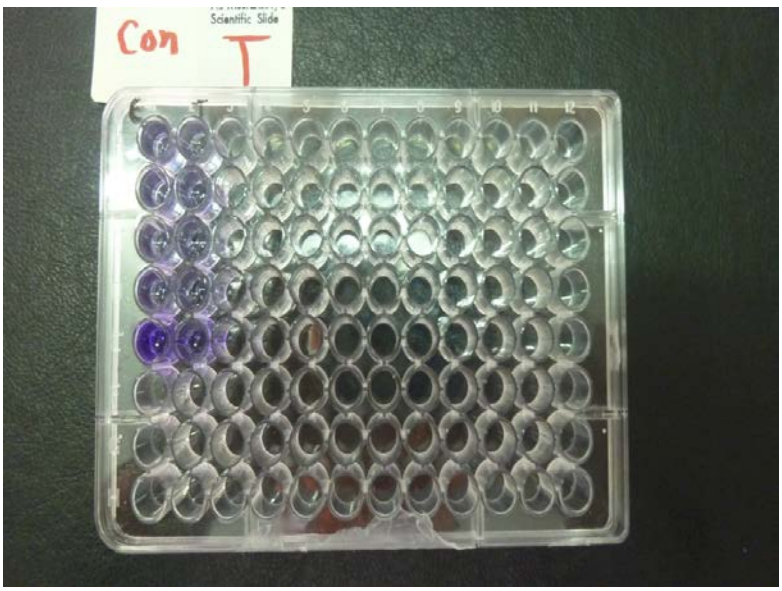


Figure3: Biofilm formation of *5 bacterial isolates* after 24h of growth in the presence of titanium nanoparticles (16mg/ml),CON:biofilm formation absesent nanoparticle

In this study, we have investigated the effects of sub-MICs of TI-NPS (Fig4.) on biofilm formation by congo red agar plates . a slime-producing strain that formed black colonies on Congo red agar plates(control) formed pink or pale gray colonies in the presence of this sub-MIC dilution of TI-NPS, thus indicating a loss of slime-producing ability for three isolates included *K. pneumonia , Morganella morganii* and *S. aureus* .while black colonies formed by *Acinetobacter baumani. & E.coli.* .Taylor&Webster *(2009)* found use of superparamagnetic iron oxide nanoparticles (SPION) as a multifunctional platform to prevent biofilm formation by *S.epidermidis* when exposed to 100 µg/ml of SPION for 12hour.

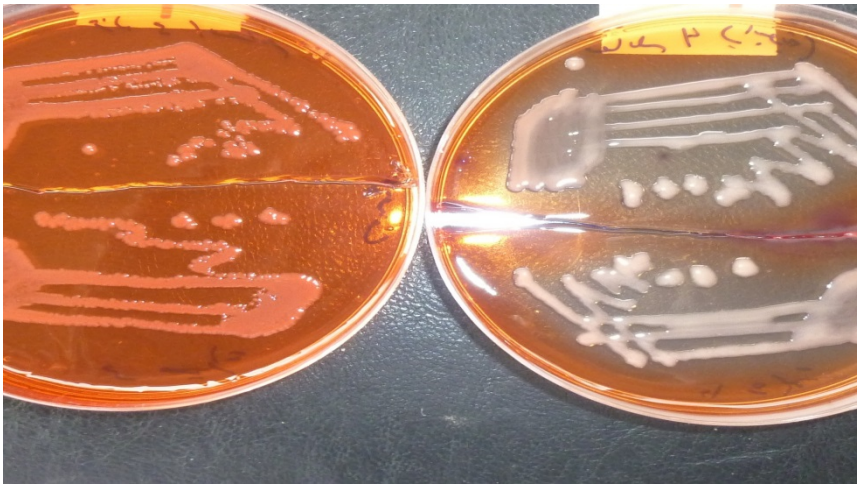


Figure4 : Effect of TI-NPS on biofilm formation on congo red agar plates assay

Nanotechnology is providing new ways to manipulate the structure and chemistry of surfaces to inhibit bacterial colonization. Nanoscale particles have emerged as novel antimicrobial agents owing to the high surface area to volume ratio, which was coming up as the current interest for researchers due to the growing microbial resistancesagainst metal ions, antibiotics and the development of resistant strains.Metal oxide nanoparticles, especially TiO2 and Ag2O nanoparticles have demonstrated significant antibacterial and antileishmanial effects(Allahverdiyev.*et al*.,2011). The antibacterial activity of TI-NPS isolated from *L. crispatus* was determined by measuring the growth obtained for some bacteria causing recurrent UTI . From those results, the MIC for each bacteria was determined, the minimum concentration (MIC) of TI-NPS was found to be 32mg/ml against all five isolates . Jha *et al*.(2009) reported that the synthesized TiO2 NPs using Lactobacillus sp. and Sachharomyces cerevisae possessed anti-bacterial and anti-fungal properties. The antibacterial activity of TiO2 has been found to be due to a reaction of the TiO2 surface with

water. On exposure to ultraviolet irradiation, TiO2 releases free radicals such as OH, O2, HO2 , and H2O2. This potent oxidizing power characteristically results in case of bacteria and other organic substances (Roy *et al*.,2010).

In the other hands Jeng *et al*.,(2006) found The bactericidal effect of TiO2 generally has been attributed to the decomposition of bacterial outer membranes by reactive oxygen species (ROS), primarily hydroxyl radicals (OH), which leads to phospholipid peroxidation and ultimately cell death . Moreover, nanoparticle binding to cell membrane or cell membrane proteins through electro-static interactions disrupting bacteria functions is another possible mechanism.

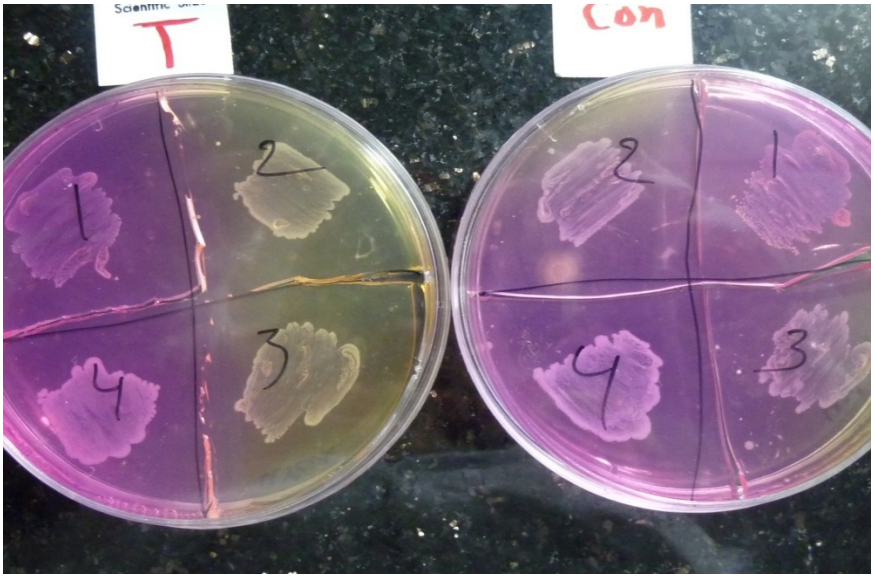


Figure 5 : Effect of TI-NPS on urease activity

Urease (urea amidohydrolase) is a hydrolytic enzyme responsible for catalytic decomposition of urea into volatile ammonia and carbamate. It is the most common enzyme used for heavy metal inhibition studies Bacterial urease is implicated in the pathogenesis of many clinical conditions. It is directly associated with the formation of infection stones and contributes to the pathogenesis of pyelonephritis, ammonia encephalopathy, hepatic coma, urinary catheter encrustation and peptic ulceration. urease is present in outer membrane (surface localized), microbial urease appear to be cytoplasmic proteins as appeard in(fig 5) our experiments showed significant decreasing in urease activity towards *E.coli&Acinetobacter baumani remaining yellow colonieswithout urea hydrolysis to ammonia &CO2.*

TiO2 is a commonly used semiconductor photocatalyst and TiO2 NPs are the most studied for photocatalytic antimicrobial activity among various NPs. A new study reported the TiO2 NPs' antibacterial efficiency in the

order of E. coli, P. aeruginosaN S. aureus. E. faecium C. albicans, is seemingly determined by the complexity and the density of the cell membrane/wall . It was also reported that the photocatalytic antimicrobial efficiency of TiO2 NPswas in the order of virus,bacterial wall,bacterial spore, depending on the thickness of microbial surface structure . Growth inhibition of Enterobacter cloacae by UVA irradiated TiO2 NPs was less effective than that of E. coli and P. Aeruginosa (choi et al.,2007)

Figure 6:Effect of TI-NPS on hemolysin Activity

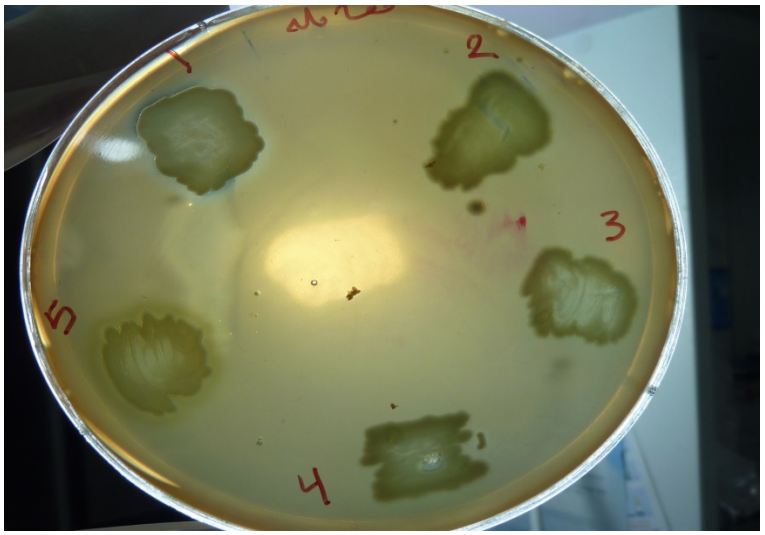


Figure7 :Effect of TI-NPS on hemolysin Activity

Titanium nanoparticles exhibited agreat effect against bacterial hemolysin activity in our work when all isolates became non beta hemolysis

on blood agar medium except two isolates *s.aureus(no 1) and morganella morganii.(no2)*

Conclusion

We reported a simple biological extracellular, easy, low cost, non toxic economical and ecofriendly approach for synthesizing titanium nanoparticles by using *Lactobacillus. crispatus*, which provides extraordinary opportunities to improve materials and medical devices. The titanium nanoparticles formed were characterized by AFM. The smallest size of the particles (70.98nm) was obtained from vaginal isolate (V20) also we showed that titanium nanoparticles biosynthesis from *lactobacillus crispatus* had antibacterial, antibiofilm ,antiadhesive,urease &hemolysis activity inhibitors properties against some bacteria causing recurrent UTI including *K. pneumonia, E. coli , S. aureus* and *Acinetobacter baumani.*

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