



Inhibitory Effect of Bismuth Oxide Nanoparticles Produced by *Bacillus licheniformis* on Methicillin-Resistant *Staphylococcus aureus* Strains (MRSA)

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Background: Based on the increase in antibiotic-resistant pathogens, it is necessary to have various effective compounds, so as to prevent its proliferation of these pathogens. For this purpose, nano-materials such as bismuth oxide nanoparticles can be used.

Objectives: The aim of this study was to produce bismuth oxide nanoparticles by *Bacillus licheniformis* PTCC1320 and to determine the antimicrobial effects on methicillin-resistant *Staphylococcus aureus* species compared with some antibiotics.

Materials and Methods: In this study, 200 bacterial samples were collected from hospitalized patients with burn infections from the Burn Rescue Hospital, Tehran. Thereafter, 65 strains of methicillin-resistant *Staphylococcus aureus* were identified by their phenotype and genotype. A total of 92% of identified strains with the highest resistance to antibiotics were isolated. Bismuth oxide nanoparticles were synthesized by *Bacillus licheniformis* PTCC1320. FTIR spectroscopy, X-ray diffraction, and scanning electron microscopy (SEM) were used to analyze the extracellularly produced nanoparticles. Finally, the antibacterial properties of nanoparticles produced on the biofilm of some pathogens were examined.

Results: In the present study, cube-shaped bismuth oxide nanoparticles were formed in the size range of 29-62 nm. They were found to have antimicrobial activity on 16% of the isolated *Staphylococcus aureus* strains. The FTIR results showed the vibrational frequencies of bismuth oxide at 583, 680, 737, and 1630 nm. The XRD results also confirmed the structure of nanoparticles. Compared with antibiotics such as Ciprofloxacin, bismuth oxide nanoparticles had less affectivity on this resistant hospital pathogen. Increasing the concentration of bismuth oxide nanoparticles, increased its antimicrobial effect and decreased bacterial growth rate.

Conclusion: Compared with heavy metals, bismuth nanoparticles have very low antibacterial effects. Considering this feature, the use of less antibiotics can be achieved with bismuth nanoparticles in the treatment of infections, thereby reducing antibiotic resistance.

Key words: *Bacillus licheniformis*, Methicillin-Resistance *Staphylococcus aureus*, Nanoparticles

1. Background

The Gram-positive bacterium, Methicillin-resistant *Staphylococcus aureus* (MRSA), exhibits resistance to the beta-lactam antibiotics through horizontal gene transfer and natural transmission (1). The antibiotic resistance of *S.aureus* strains has become a global warning problem, resulting to failure in the treatment of bacterial infections, thus endangering the patient's life and increasing the cost of treatment. This microorganism is regarded as a commonly occurring infectious disease which cause the formation of biofilms in damaged

tissue and patients hospitalized with catheters (2).

MRSA is responsible for common infections in hospitals. Patients with weak immune systems, Open injuries, diabetes disease, catheters diseases and also, the elderly are exposed to more risk. MRSA strains are the main cause of sepsis in hospitals around the world (3, 4). PCR-based search of the *mecA* gene is the most accurate method for MRSA detection (5). Microorganisms are linked to various surfaces through the Exo-polysaccharide Matrix (EPS) to create antibiotic resistance. In fact, the EPS matrix protects microbial

cells from high concentrations of antibiotics which leads to chronic infections despite antibiotic treatments. Different groups of bacteria form biofilms which cause very important problems in medicine and industry. The growing microbial cells in biofilms are physiologically different from their planktonic counterparts (6). In 2010, the American community urged the discovery of new drugs to fight antibiotic-resistant microorganisms, stating that one of the promising strategies for overcoming microbial resistance is by making use of nanoparticles (7). Microorganisms use the organic and inorganic resources in the environment to perform their metabolism and critical processes. When exposed to metal ions, microorganisms accumulate these materials inside or on the cell walls through different processes. This accumulation often results in the production of particles that are packaged in nanoparticles (8). Inorganic nanoparticles are used in various medical fields to diagnose and treat diseases (9). In recent years, nanotechnology has yielded amazing growth and development in nanoparticles-based medicine which has been a revolution in the treatment of diseases by potent and less toxic drugs (10). In 2013, Nazari *et al.* synthesized bismuth by *Serratia marcescens* (11) but in this study, Bismuth oxide nanoparticles were produced using *Bacillus licheniformis* PTCC 1320 strain for the first time and its antimicrobial effect was investigated on methicillin-resistant *Staphylococcus aureus* biofilms. There has been no study on bismuth oxide nanoparticles produced by *Bacillus licheniformis* PTCC 1320 but bismuth has been synthesized by *Serratia marcescens* (11).

2. Objective

The aim of this study was to produce bismuth oxide nanoparticles using *Bacillus licheniformis* by a green method, and the investigation of its antimicrobial effect on methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from hospitalized burn patients .

3. Materials and Methods

3.1. Preparation of *Bacillus licheniformis* PTCC 1320 With Bismuth Nitrate Resistance

Bacillus licheniformis PTCC1320 was obtained from the Iranian Research Organization for Science and Technology, Tehran, Iran.. The bacteria was cultured on a bismuth sulfide agar medium (MERCK, Germany) and incubated at 30 °C for 24 h, 0.2% w/v bismuth nitrate solution was added to the nutrient agar (MERCK, Germany). The standard *Bacillus* strain was grown at 30 °C for 24 h.

3.2. Synthesis of Bismuth Oxide Nanoparticles

Bismuth nitrate $\text{Bi}(\text{NO}_3)_3 \cdot 5\text{H}_2\text{O}$ with a final concentration of 485 g.L^{-1} was prepared in distilled water. In the present study, 0.003, 0.055, 0.03 and 0.05 M of Bismuth nitrate were prepared for use in subsequent syntheses. The bacterial strains were incubated in 50 mL of a salt-free broth, with MacFarland half-concentration at 35 °C for 24 h. This was followed by centrifugation for 20 min at $8000 \times g$ and 4 °C, the biomass was discarded. The supernatant was studied for the extracellular production of bismuth oxide nanoparticles. The bismuth nitrate solution was added to the supernatant with a 10% V/V ratio and incubated at the same temperature. After color change, the sediment was separated by centrifugation at $8000 \times g$ in 20 min (11, 12).

To ensure that other additional items were completely removed, the sediments were washed three times with 0.9% w/v NaCl solution. The precipitate was incubated for 2 to 3 days at 40 °C. Then, in order to carry out calcination of the nanoparticles, the resulting precipitate was placed in a furnace for 2 h at 550 °C (13). Bismuth oxide nanoparticles were then analyzed using FTIR spectroscopy, X-ray diffraction (XRD) and scanning electron microscopy (SEM).

3.3. Sample Collection of Hospitalized Patients

Bacterial samples (200 samples) were collected from wound infections of burn patient injuries from Motahari Hospital in Tehran, starting from October, 2016 to July, 2017. Sampling was performed using a cotton swab impregnated in normal saline. In order to identify methicillin-resistant *Saureus*, the specimens were cultured in the Mannitol Salt agar medium (MERCK, Germany) and incubated at 37 °C for 48 h. Then, sixty-five strains of *Saureus* resistant to antibiotic-resistant methicillin were identified.

3.4. DNA Extraction of Methicillin-Resistant *Staphylococcus aureus*

After the morphological identification of strains and biochemical tests (Gram staining, catalase, coagulase, DNase, and growth on mannitol salt agar media), gene sequencing was used for genus identification (14). Single bacterial colonies were inoculated into a 5 mL suitable culture medium (LB), shaken for 24 h at 37 °C and 120 rpm. Genomic DNA was extracted using Genom DNA extraction kit, according to the manufacturer's instructions (Cinnagen, Iran). The extracted specimen was stored at -20 °C.

3.5. PCR-Based Genotyping Assays

Forward and reverse primers with a length of 140 bp were designed for the molecular detection of *mecA* (15)

Table 1. The sequences of primers used in this study.

Gene	Forward primer	Reverse primer	Lenght (bp)
<i>mecA</i>	AACAGGTGAATTATTAGCACTGTAAG	ATTGCTGTTAATATTTTTTGAGTTGAA	140 bp

using PCR (**Table 1**). The PCR process was done to a final volume of 25 μL PCR by mixing 18 μL of double distilled water, 2.5 μL of PCR buffer (10X), 1 μL of dNTP (10 mM), 1 μL of Forward primers (10 PmoL) and 1 μL Reverse Primers (10 PmoL), 1 μL of genomic DNA (100 ng) and finally, 0.5 μL of enzyme Taq Polymer (1U.100 μL^{-1}). PCR was performed with an initial denaturation step 94 $^{\circ}\text{C}$ for 5 min, followed by 30 cycles of 94 $^{\circ}\text{C}$ for 1 min, 55 $^{\circ}\text{C}$ for 1 min and 72 $^{\circ}\text{C}$ for 2 min, with a final extension at 72 $^{\circ}\text{C}$ for 10 min. Then samples were run on 1% agarose gel based on TAE buffering conditions at 80 v for 1 h. To determine the size of the amplified fragment, a 100 bp marker was used. The gel was then stained with ethidium bromide solution for 10 min. The gel was visualized using a UV illuminator.

3.6. Resistance Study of Strains to Antibiotics

In order to investigate the lack zone of growth and determine sensitivity to antibiotics, all strains were concentrated on the Muller Hinton Agar culture medium (MERCK, Germany).

Then by using the Kirby-Bauer method, according to the Clinical Laboratory Standards Institute (CLSI) table, sensitivity to the antibiotics of microorganisms was investigated. Antibiotics such as ciprofloxacin (5 μg), vancomycin (30 μg), gentamicin (10 μg), ceftazidime (30 μg), cefalotin (50 μg), tetracycline (30 μg), methicillin (5 μg), rifampin (5 μg) were used (16).

3.7. Disc Diffusion Method for Studying the Antimicrobial Properties of Bismuth Oxide Nanoparticles

In order to investigate the antimicrobial effect of the bismuth oxide nanoparticles produced by *B. licheniformis* PTCC 1320, 1mL of methicillin-resistant *Staphylococcus* bacteria was added to the Muller Hinton Agar (MERCK, Germany); then it was cultivated with a sterile swab. Blank disks were prepared with a diameter of 6 mm. Thirty sterile blank disks were kept for 1 h to complete the absorption in different dilutions of bismuth oxide nanoparticles i.e. 100, 300, 600, 900, 1200 and 1500 ppm. Then they were placed at 40 $^{\circ}\text{C}$ for 1 h to dry. They were placed in a muller Hinton agar culture medium containing the mentioned pathogens, with the appropriate distance from each other and the edge of the plate. Finally, the plates were incubated at

37 $^{\circ}\text{C}$ for 24 h. After which the halo zone diameter was measured (17).

3.8. MIC (Minimum Inhibitory Concentration)

To examine the minimum inhibitory concentration (MIC) for bacterial growth using serial dilutions were prepared from the solutions of Bismuth oxide nanoparticles, a different volume of Mueller-Hinton broth medium (MERCK, Germany), and inoculated bacterial suspension. MRSA with the concentration of 1.5×10^8 CFU.mL⁻¹ were added separately to 20 mL cultures in five conical flasks (50 mL). The cultures were incubated at 37 $^{\circ}\text{C}$ with shaking at 150 rpm for 24 h. Growth rates and bacterial concentrations were observed, and Broth Dilution Method were used (18).

3.9. Biofilm Formation

From single culture colonies of methicillin-resistant *S.aureus* strains and the liquid control in the LB medium with the standard 0.5 Mac Farland at a wavelength of 630 nm for each sample was set. Then, 100 μL of each sample and the control strain were added to 6 parallel pits in a 96-well polystyrene microtiter plate. Also, the LB medium (MERCK, Germany) with 1% glucose in a 6-well row was used as control. The samples were incubated at 37 $^{\circ}\text{C}$ for 48 h. The 0.01 M isotonic potassium phosphate buffer was added to each well with sterile saline (pH 7.5). Then about 200 microliters of pure methanol was added to each well and placed at room temperature for 10 min. The crystalline violet color was then added at 1% (W/V) and kept for 20 min at room temperature. After evacuation and washing with water, 33% glacial acetic acid was added. Each well was analyzed using an ELISA reader at 630 nm. Each test was repeated three times (19).

3.10. Antimicrobial Characteristics of Bismuth Oxide Nanoparticles

Ciprofloxacin 0.5 mg.L⁻¹ was filtered then the effect of 1500 ppm bismuth oxide nanoparticles and Ciprofloxacin on antibiotic resistant isolates strains from hospitalized patients was investigated. For this purpose, 96 microtiter plates containing live biofilms were used to investigate the antimicrobial properties of bismuth oxide nanoparticles and incubated at 37 $^{\circ}\text{C}$ for 24 h. Then the biofilms OD were evaluated. Luria-

Bertani (LB) culture medium OD was analyzed without biocide and with monoclonal bacteria as control (12, 17).

4. Results

4.1. Biosynthesis of the Bismuth Oxide Nanoparticles (Bi_2O_3 NPs)

After the cultivation of *B.licheniformis* PTCC 1320 for 24 h, the colonies appeared to be black with metal lining. At the 0.005 M concentration of bismuth oxide, the supernatant was changed (from yellow to brown) after 7 days and it was investigated for the presence of nanoparticles (**Fig. 1**).

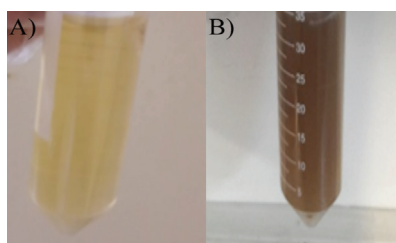


Figure 1. Change of colors after 7 days of incubation. A: culture supernatant with bismuth nitrate, B: supernatant mixed with Bi_2O_3 NPs.

4.2. Identification of Microorganism

Sixty-five isolated bacteria from hospitalized patients with burn infections was identified following to the routine biochemical and morphological examinations. Results showed that the isolated strain relates to the methicillin-resistance *Staphylococcus aureus* species. The isolated strain was cocci-shaped and Gram-positive bacterium. As well the bacterium was found to be Catalase, coagulase, DNase positive and Oxidase negative. Yellow colonies growth on mannitol salt agar (14).

In this study, PCR amplification showed a length of about 140 bp of the product, MRSA strains were positive for the presence of *mec A* gene with the highest antibiotic resistance. (**Fig. 2**).

The SEM results showed that the bismuth oxide nanoparticles produced had a uniform and cube-shaped morphology (**Fig. 3**). Average particle size of Bi_2O_3 was about 45 nm.

B.licheniformis strain 1320 has also been shown to produce extracellular BiNPs with corresponding crystalline surfaces. ; According to the Debye Scherrer formula, they had an average size of 47.47 nm.

All nanoparticles produced were smaller than 100 nm, as it is clearly shown in the SEM image (Fig.3).

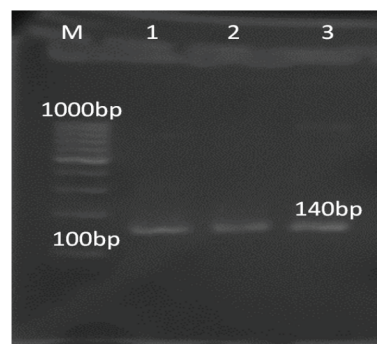


Figure 2. Electrophoresis of *mecA* 140 bp genes on 1% agarose gel. Lane 1-3: PCR product of *mecA* gene (140bp); M: 100 bp DNA size marker.

According to the Debye Scherrer formula, the average size was 47.47 nm. All nanoparticles produced were smaller than 100 nm, as it is clearly shown in the SEM image. Assignments of Miller indices (hkl) are presented in Table 2, a family of lattice planes was determined by three integers h, k, and l, the Miller indices and denotes the family of orthogonal planes, which is the basis of the reciprocal lattice vectors.

X-ray diffraction patterns and FTIR spectrum demonstrated that the purified NPs consisted of Bi-O-Bi in wavelengths of 583 cm^{-1} , 737 cm^{-1} stretching vibrational of O-Bi-O, and 1630 cm^{-1} stretching of Bi-O and 3437 cm^{-1} stretching vibrational of O-H group for hydroxyl functional group in surface of Bi_2O_3 (Figs. 4 and 5).

4.3. Antibacterial Activity

The highest antibiotic sensitivity rates were reported

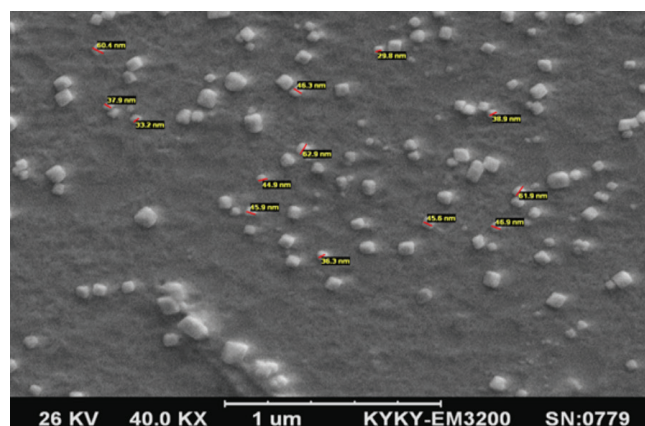


Figure 3. SEM image of Bismuth oxide nanoparticles produced by *B.licheniformis* Strain 1320.

Table 2. XRD Results showing the compliance of Bismuth Oxide with the Standard peaks.

	Observed 2θ	Observed Intensity	(hkl)	Standard 2θ	Standard Intensity
XRD major peak of Bi ₂ O ₃	27.33	100	-121	27.38	100
	33.17	70	-202	33.23	36
	52.37	87	-322	52.38	12
	46.34	69	-223	46.29	18
Crystal size by Sherrer equation				47 (nm)	
Particle size by SEM				45 (nm)	

hkl: Miller indices

for ciprofloxacin 85% while the lowest was reported for methicillin (8%), rifampin (4%) and vancomycin (3%). The highest concentration of Bi₂O₃NPs (1500 ppm), showed antibacterial activity Approximately similar to gentamycin (15%) and cephalothin (15%). **Figure**

6 shows the percentage of MRSA strains sensitive to antibiotic and bismuth oxide nanoparticles.

The results showed that the produced nanoparticles with a concentration of 1500 ppm and the inhibitory effect of 17 mm had the highest inhibitory effect on the

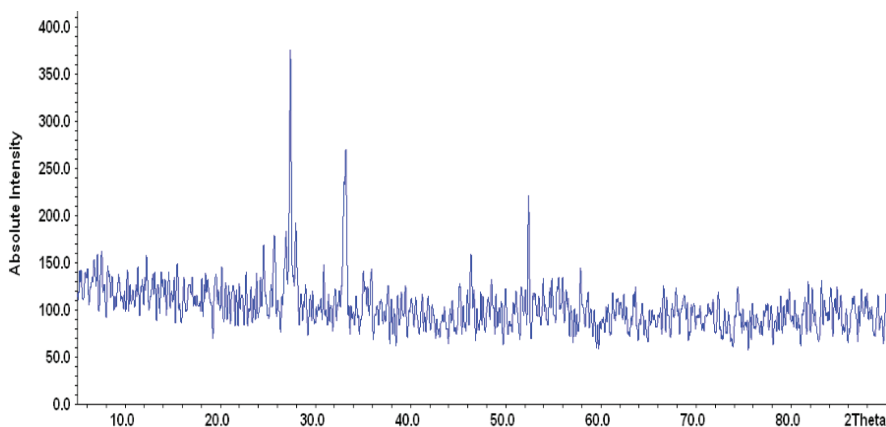


Figure 4. X-ray diffraction patterns derived from bismuth oxide nanoparticles produced by *Bacillus licheniformis* PTCC 1320.

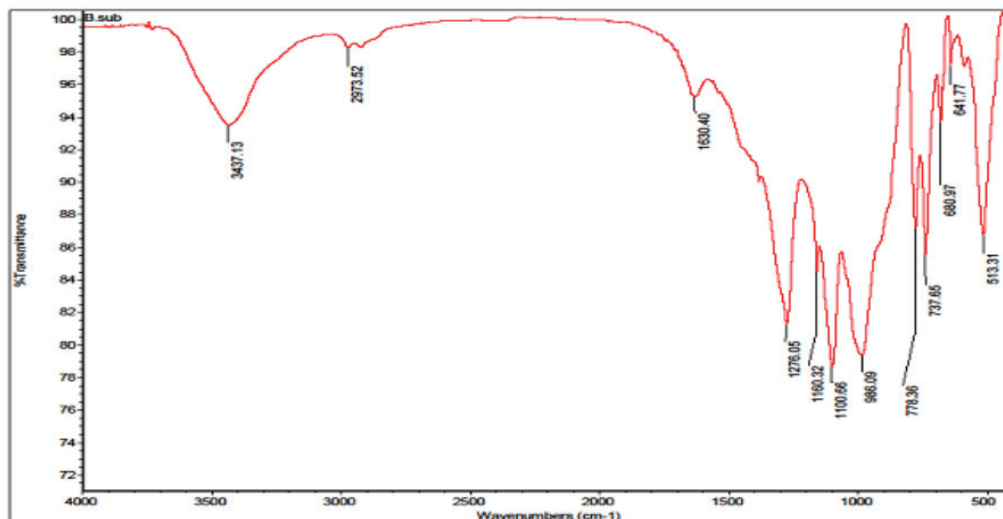


Figure 5. FTIR spectrum of Bi₂O₃ nanoparticle synthesized by *Bacillus licheniformis*.

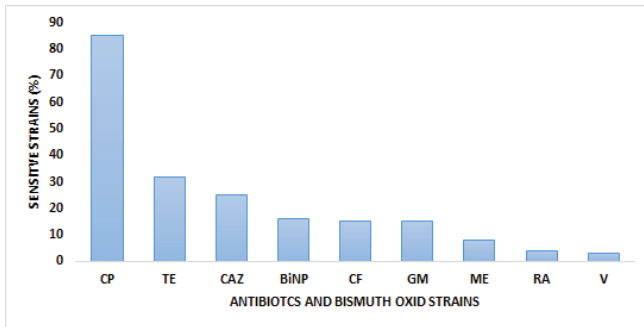


Figure 6. The sensitivity of MRSA strains to patients hospitalized with burns. (CP) ciprofloxacin, (TE) tetracycline, (CAZ) ceftazidime, (BiNP) bismuth nanoparticles, (GM) gentamicin, (CF) cephalothin, (ME) methicillin, (RA) rifampin, (V) vancomycin.

MRSA strains, while the concentration of 100 ppm with the lowest inhibitory effect recorded the least effect.

4.4. Biofilm Formation

The average absorption of the *Staphylococcus aureus* biofilm was recorded at 1.557 nm. Then, in the presence of ciprofloxacin and bismuth oxide nanoparticles, OD was read with an average of 0.65 and 1.055 for methicillin-resistant *S.aureus*, respectively. The OD of the negative control was 0.04, and the positive one was 1. Bismuth oxide nanoparticles had an inhibitory effect on 16% biofilm of the pathogen samples while in planktonic conditions, all sixty-five strains were inhibited by increasing the nanoparticles concentration. It could be concluded that biofilm formation produced drug resistance in the bacteria.

5. Discussion

Nanotechnology is a new field with many uses in medicine, for example the treatment and diagnosis of disease. Nanoparticles have antibacterial activity like that of silver, copper oxide, bismuth and selenium. The advantages of inorganic nanoparticles include high surface-volume ratio, different shapes, unique structural forms and nanoparticle sizes which interact with bacteria. These characters among nanoparticles and antimicrobial agents are the main difference and are capable of reducing the danger of developing antibacterial resistance. For instance, the crystalline structure of bismuth nanoparticles can react with biological systems, including bacteria, fungi and viruses. One of the most important characteristics of nanoparticles is that they reduce the probability of antimicrobial resistance development (20).

The extracellular synthesis mechanism of

nanoparticles using microorganisms is yet to be fully understood and their exact mechanism of action against biological systems are yet to be completely elucidated. Several studies have reported that a positive charge on the metal ion is acute for antibacterial activity, permitting for electrostatic absorption between the negative charge on the bacterial cell membrane and the positive burden on the nanoparticle. Bismuth oxide is used in the production of glass, ceramic products and catalyst in the oxidation of hydrocarbons (21).

In this study, *S.aureus* was used to study the effect of bismuth oxide nanoparticles produced extracellularly through the use of *B.licheniformis* PTCC 1320, with a change in the color of the supernatant from yellow to brown, as the selected strain, the green method was selected to form the nanoparticle and its formation was confirmed by infrared spectroscopy, XRD and SEM. The results of this study indicate that cubical and uniform Bi_2O_3 NPs (29-62 nm) were produced using *B. licheniformis*. These NPs had effective antibacterial property against MRSA but compared with antibiotics, bismuth oxide nanoparticles had a low effect on this pathogen. The MIC of bismuth oxide nanoparticles for bacterial growth inhibition was 1500 ppm. Compared to heavy metals, bismuth nanoparticles have very low toxic effects. Considering this feature, it may be possible to reduce the concentration of antibiotics and the use of bismuth oxide nanoparticles in the treatment of infections, results in decreasing antibiotic resistance. To determine if bismuth oxide nanoparticles can interfere with MRSA biofilm formation, the antibiofilm activity of these nanoparticles was evaluated. The observation of antibiofilm activity of Bi_2O_3 NPs against the MRSA showed the bactericidal activity of the nanoparticles against 16% pathogenic bacteria and nanoparticles have antibacterial activity against the planktonic form of MRSA in high concentration. The total bactericidal activity has been determined in different concentrations of the Bi_2O_3 NPs but at lower concentration the inhibition rate was low.

Hernandez–Delgado *et al.* studied the inhibitory effect of chemically synthesized bismuth zerovalent nanoparticles on the formation of *Streptococcus mutans* biofilms. Growth inhibition of these bacteria was observed (20). On the other hand, Nazari *et al.* studied the antimicrobial activity of bismuth nanoparticles produced by *S.marcescence* (isolated from the Caspian Sea) on *Helicobacter pylori*. Bismuth nanosized particles were smaller than 100 nm when synthesized. They showed that bismuth nanoparticles had a antimicrobial activity on these strains (11), which is similar to this study Which indicates the antibacterial

activity of bismuth nanoparticles.

In 2015 and 2016, Delijou *et al.* (22) and Sarangadharan *et al.*(23), respectively, synthesized silver nanoparticles, Shivashankarappa (24) synthesized Cds nanoparticle while Thakur *et al.* (25) synthesized gold nanoparticles through the use of *B. licheniformis* in 7-31, 3-170, 20-40, and 45 nm particle sizes, respectively. This study showed the ability of *B.licheniformis* to synthesize Bi₂O₃NPs in the range of 29-62 nm size. The results of earlier studies and the present study indicate the potential of *B.licheniformis* bacteria in the synthesis of nanoparticles in good range and different sizes of nanoparticles perhaps for use of different doses of precursor or type of precursor. To the our knowledge, this is the first report of bismuth oxide nanoparticles synthesized by the *B.licheniformis*.

Biofilm isolates of the isolated MRSA strains from patients were studied using PCR molecular methods and the effect of the above nanoparticles on the planktonic and biofilm bacteria was evaluated and compared with the antimicrobial effect of ciprofloxacin antibiotic. This study showed that Bismuth oxide nanoparticles were effective in increasing the concentration against planktonic form, but their anti-biofilm activity was low when compared with ciprofloxacin The size and shap of nanoparticle may influence against bacteria (22). The advantages of the bacterium to the production of Bismuth oxide nanoparticles are high availability, low-cost production, and GRAS microorganism.

The results of this study showed that, compared with antibiotics, bismuth oxide nanoparticles had a low effect on this pathogen. Compared to heavy metals, bismuth nanoparticles have very low toxic effects. Considering this feature, it may be possible to reduce the concentration of antibiotics and the use of bismuth oxide nanoparticles in the treatment of infections, results in decreasing antibiotic resistance.

At the end, it is suggested that, based on studies, bacteria such as *S.marcescence* and *B.licheniformis* are capable of synthesizing bismuth nanoparticles, which can be used to synthesize other nanoparticles in the industry and medicine to battle bacterial resistance, and also, the quantity and time of production of nanoparticles are compared.

Producing metal nanoparticles using the bacteria, the mechanism of conversion of metal ions into nanoparticles is not understood completely. Therefore, it is suggested that this mechanism would be investigated, this might lead to more optimized methods of producing these nanoparticles.

Biosynthesis of bismuth nanoparticles with *Bacillus* bacteria and investigating their abilities in medical and

pharmaceutical industries can be evaluated. Overall, the experimental results suggest that these nanoparticles could be an interesting alternative to fight the infections underlying planktonic form. But further experiments are required for use in the human body.

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