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Biosynthesis of bismuth nanoparticles and its synergistic effect with antibiotics against Escherichia coli and Klebsiella pneumoniae

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ABSTRACT

Introduction. Today, the biosynthesis of nanoparticles (NPs) assisted by microorganisms (particularly bacteria) received increasing attention. In this study, *Bacillus subtilis* strain SFTS, a bismuth-reducing bacterium, was isolated from the soil of a copper mine in the South of Iran and used for biosynthesis of bismuth NPs (Bi NPs).

Materials and methods. *Bacillus subtilis* strain SFTS was identified by conventional identification tests and the 16S rDNA fragment amplification method. Characterizations of the bio-fabricated Bi NPs were examined using FTIR, EDS, XRD, TEM, and SEM analysis after purification of Bi NPs. In addition, the synergistic effect of biogenic Bi NPs in combination with different antibiotics was also investigated.

Results. The attained results revealed that the biosynthesized Bi NPs average size was 22.36 nm and spherical in shape. The XRD pattern showed that the biosynthesized nanoparticles consisted only of Bi_4 and monoclinic crystals. Furthermore, the results of antibacterial effect of Bi NPs in combination with various antibiotics showed that the nanoparticles represented the highest synergistic effect together with imipenem and the lowest effect in combination with tetracycline against clinical strains of *E. coli* and *K. pneumoniae*. Significant difference between synergistic effect of Bi NPs with antibiotics compared to antibiotics disc alone against *E. coli* and *K. pneumoniae* strains was observed (P<0.001).

Conclusion. This study showed that Bi NPs biologically synthesized by *Bacillus subtilis* strain SFTS had a small size and different structure. However, finding about their antibacterial effect and related mechanism merit further investigations.

Keywords: Bismuth nanoparticles, Bi NPs, Biogenic nanoparticles; Biosynthesis; Antibacteria

Introduction

Nanotechnology plays an important role in the development of different areas. In certain, nanoparticles (NPs) have been extensively used in diverse fields, varying from engineering to biomedicine, because of their unique size-dependent physicochemical properties (for example, elevated surface-to-volume ratio) [1]. Nanoparticles displaying antibacterial effects can aim at multiplex biomolecules and have the possibility to decrease or stop the evolution of multidrug-resistant organisms (MDROs). In this respect, the usage of nanoparticles gives a potential strategy to control infections created by MDROs and showed therapeutic promise [2].

Despite the capability of chemical and physical approaches to synthesize nanoparticles of special shape and size, application of dangerous matter

and non-affordable property of these methods make their application limited [3]. However, the applicable effects of metal nanoparticles can be enhanced by the biogenic synthesis strategy. Biogenic synthesis of nanoparticles is look for notable attention owing to the reality that it is environmentally friendly compared to other ways of nanoparticle synthesis [3, 4]. In addition, reproducibility of synthesized nanomaterials and stability create biogenic synthesis a preferred procedure over the other ways [3]. The biogenic approach is better supported by the fact that the majority of bacteria inhabit environmental conditions of different temperature, pH, and pressure [5]. Employment of bacteria in the biosynthesis of nanoparticles has advantages like the ability to create sustainable nanoparticles at a large scale [6]. Such improved stability might attributed to the coating of the nanoparticle surface by microbial proteins and different biomolecules [7]. Other advantages of using bacteria for the biosynthesis of nanoparticles can be mentioned as simple reproduction and its ability to fabricate nanoparticles with uniform sizes [8].

Bismuth with the symbol of Bi (lethal dose > 5–20 g/day/Kg, to years) is a metallic element that its combinations have been utilized in treatment for more than 200 years [9]. Several Bi combinations show antibacterial activity [10] and are able to act better than the inhibitory activity of antibiotics [9, 11]. On the other hand, the antimicrobial effects of Bi can be enhanced by nanotechnological methods cause of their particular structural features [9]. Several studies have demonstrated the synergistic effect of Bi NPs in combination with different antibiotics against bacterial pathogens [12, 13]. Hence, this study aimed to screen and identify new bacterial isolate for biosynthesis and characterization of Bi NPs and combine these nanoparticles with different antibiotics against clinically isolates of *E. coli and K. pneumoniae.*

Materials and methods

Bacterial strains and chemicals

Two standard strains of *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 13883 and two clinical isolates of *E. coli* and *K. pneumoniae* were isolated from clinical specimens admitted to Afzalipour hospital in Kerman, Iran, were used. Bismuth subnitrate [$Bi_5O(OH)_9(NO_3)_4$], nutrient broth (NB), nutrient agar (NA), Muller Hinton agar (MHA), Muller Hinton broth (MHB), dithizone ($C_{13}H_{12}N_4S$), ethanol, sodium dodecyl sulfate (SDS), Tris-base, and n-octyl alcohol were bought from Merck Chemicals company (Darmstadt, Germany). In addition, the antibiotic discs were used from Padtan teb Company (Padtan teb, Iran).

Isolation and identification of Bi NPs biosynthesizing microorganism

The bacterial isolate utilized in the current study was separated from the soil sample of a copper ore mine in the Jiroft region, Kerman province in south-east of Iran. The soil sample was collected in 15 ml sterile falcon and 10 ml of sterile normal saline was added and mixed, followed by transferring of some resulting suspension and incubating on nutrient agar medium for 48 h at 37 ºC. Subculturing was carried out for obtaining single colonies. Separately, different single colonies were cultured on a nutrient agar medium containing bismuth subnitrate, and a colony that altered the color of the medium culture from yellow to dark-brown was indicated as a bacterium synthesizing Bi NPs for the next steps. Identification of selected microorganism was performed according to the current morphological and biochemical assays (Gram staining, cell morphology, motility, catalase test, oxidase test, lactose and glucose fermentation assay, urease test, indole test, and H_2S production) according to Bergy's manual determination of bacteriology [14]. In addition, to complete the isolate identification, 16S rDNA gene sequence investigation was used to compare it with sequences shared in GenBank of NCBI. Briefly, after 24 h cultivation of the desired isolate in NB medium, the produced biomass was separated using centrifugation for 5 min with 11000 g, then the cells were washed three times with deionized water. The genomic DNA was obtained by boiling the cell suspension for 10 min at 94 °C and then it was centrifuged for 5 min at 11000 g. Amplification of bacterial 16S rDNA gene was carried out by applying the universal forward (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse (5'-TACGGTTACCTTGTTACGACTT-3') primers [15] using a thermal cycler (PEQLAB, Erlangen, Germany) which was programmed as follows: 1) initial denaturation for 3 min at 94 °C, 2)

30 cycles of denaturation for 1 min at 94 °C, annealing for 45 sec at 55 °C, and synthesis for 90 sec at 72 °C. and, 3) final extinction for 5 min at 72 °C. Consequently, the amplified DNA fragment was purified on 1% agarose gel in 1x TAE buffer and shipped for sequencing utilizing the noted primers (Sinaclon Incorp., Iran). The acquired sequence through the basic local alignment search tool (BLAST) was aligned to compare with the sequences deposited in GeneBank of NCBI.

Growth curve of Bi NPs synthesizing bacteria

To examine the growth curve of selected bacterial isolate, the desired bacterial strain was inoculated at NB medium culture and 1 ml of inoculum with OD_{600} equal to 0.1 was added to a 500-mL Erlenmeyer flask with 100 ml of NB medium culture containing 0.1 mg/ml of Bi^{3+} ions. Then, the Erlens were aerobically cultivated for 72 h at 150 rpm and 30 °C. Also, the inoculated culture medium without bismuth ions was separately prepared to admit that the color change is not related to bacterial pigment. Furthermore, the culture medium containing bismuth without bacteria was used to investigate the reduction of bismuth in the absence of bacteria. Finally, the 1-ml samples were taken every three hours and were measured for OD_{600} [16].

Furthermore, in order to measure Bi^{3+} ion concentration in the taken samples, an amount of 500 µl dithizone solution (0.1 mg/ml in ethanol) was mixed with 500 µl of taken sample and the absorbance of related complex was recorded at 502 nm [17]. The Bi^{3+} ion concentration was then determined according the standard curve previously drawn by measurement of maximum absorbance (at 502 nm) of different Bi^{3+} ion concentrations (0.063–0.2 mg/ml) in the presence of dithizone solution. Furthermore, all investigations were taken out in triplicate, and the mean results was employed to draw bacterial growth curve and Bi^{3+} ion concentration.

2.4. Biogenic synthesis and purification steps of Bi NPs

For the first step, for the preparation of Bi NPs, 1 ml of a fresh overnight culture of the isolate was inoculated aerobically at 150 rpm and 30 °C, in 100 ml of sterile nutrient broth culture medium augmented with 2 mg/ml of bismuth subnitrate. Bacterial cells were separated from the culture medium after 72 h via centrifugation for 10 min at 4000 g. Bacterial biomass was then broken after freezing in liquid nitrogen, followed by ultrasonication (100 W, 5 min) and washing serially with centrifugation for 5 min at 10000 g, using 1) Tris-HCl buffer (1.5 M and pH 8.3), 2) SDS (1%), and 3) deionized water. The washing process was repeated three times to ensure about separation of any undesirable materials. In the next step, Bi NPs were purified by creating an organic-aqueous biphasic partition system using n-octyl alcohol [18].

Characterization analysis of Bi NPs

The UV–Vis (ultraviolet-visible) spectra of the purified Bi NPs in the wavelength spectrum of 200–700 nm was measured utilizing a spectrophotometer (UV-1800, Shimadzu Corporation, Tokyo, Japan). Using transmission electron microscopy (TEM), the morphology and size of Bi NPs nanoparticles were determined. Also, an energy dispersive X-ray spectroscopy (EDS) and a scanning electron microscope (SEM) apparatus were used to study elemental composition and the shape of the purified Bi NPs. To examine the particle size distribution, it was measured using a Zetasizer MS2000 device (Malvern Instruments, UK). Furthermore, Bi NPs were analyzed for FTIR spectra utilizing a Bruker Alpha tool (Bruker Optics, MA, USA) and for X-ray diffraction (XRD) pattern by XRD Xpert Pro Panalytical (Panalytical, Holland).

2.6 The synergistic impact of Bi NPs in mix with antibiotics

To determine the synergistic impact of Bi NPs in mix with antibiotics (imipenem, ceftazidime, levofloxacin, and tetracycline), disc diffusion approach against the clinical and standard isolate of *K. pneumonia* and *E. coli* was used. In summary, the target bacteria were cultured on a nutrient agar medium culture and incubated for 24 h at 37 °C. Then, using a loop, several colonies from the fresh culture were dissolved in sterile normal saline to get the standard of 0.5 McFarland. Afterward, the obtained suspension was cultured uniformly on Muller-Hinton agar medium by a sterile swab. Finally, the standard antibiotic discs were set on the medium alone and with Bi NP (40 µl/disc from stock of 5000 µg/ml), as well as blank discs impregnated with Bi NP and incubated at 37 °C. The diameter of the growth inhibition zones was measured after 24 h incubation [18]. All tests were

conducted three times and the average values and the standard deviation (mean ± S.D.) were reported. The one-way ANOVA analysis was used to compare the data between an antibiotic disc and an antibiotic disc containing Bi NPs, and $p<0.001$ was regarded as significant.

Results and discussion

Isolation and Identification of the Bi NPs biosynthesizing bacteria

The acquired examination of the screening stage demonstrated that one sample from the soil (isolated from a copper mine in the Jiroft region, Kerman province in south-east of Iran) included a bacterial strain was able to reduce Bi^{3+} ions to Bi^{0} which was obvious from color shift from yellow to dim brown. After cultivation of the isolate for 72 h at 30 ˚C, the colonies appeared to be brown on nutrient agar medium containing bismuth and, was changed color from yellow to brown in nutrient broth medium contains 2 mg/ml of Bi subnitrate (Fig. 1), which this color change revealed the presence of Bi NPs and is similar to other studies [19-23].

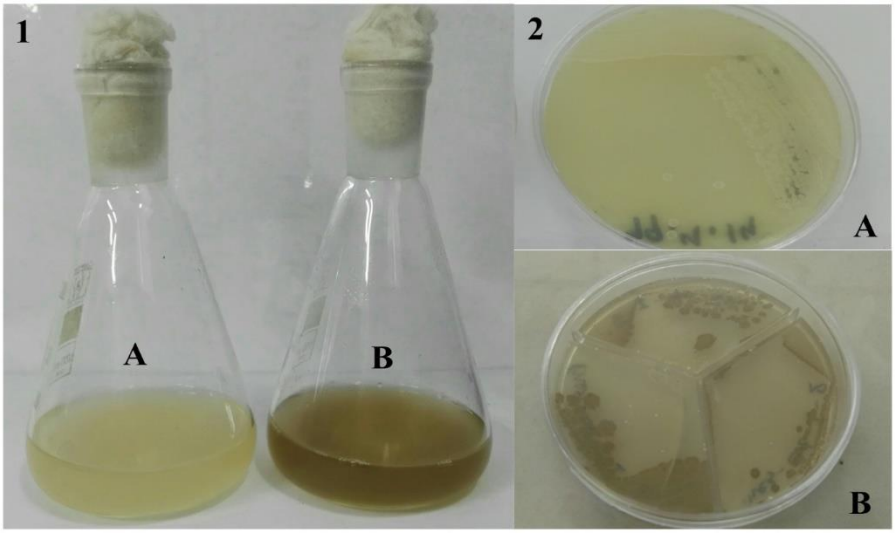

Fig 1. Change of colors after 72 h of incubation. 1: NB without (A) and with (B) bismuth subnitrate 2: NA without (A) and with (B) bismuth subnitrate with *Bacillus subtilis* strain SFTS.

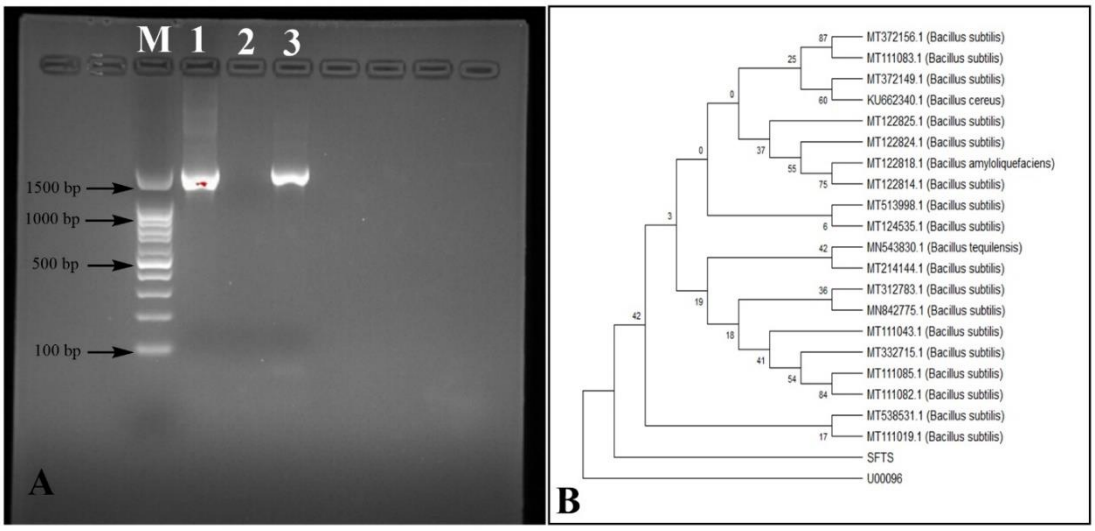

Fig 2. Electrophoresis of 16s rDNA gene product on 1% agarose gel (A). Lane M: the DNA size marker ladder, Lane 1: Positive control (*Escherichia coli*), Lane 2: Negative control (deionized sterile water), Lane 3: Sample. B: phylogenetic tree based on 16s rDNA gene sequences. 16s rDNA gene sequences.

Today, with antibiotic resistance and the side effects of their use, researchers are moving to alternative methods [24]. Biogenic nanoparticles produced with metal-reducing bacteria give advantages not shared with their chemically made equals [25]. Among the cases of biogenic nanoparticle synthesis, bacteria, because they are

easily present in the environment, can be cultured quickly and are able to adapt to different conditions, making them a good candidate for nanoparticle synthesis [26]. Due to the ability of *Bacillus* bacterial strains to create different structures of inhibitory compounds [27], in this study, we introduce a new strain of *Bacillus* synthesizing Bi NPs (*Bacillus subtilis* strain SFTS) with its properties. Limited studies have synthesized Bi NPs using bacteria, including Shakibaie et al. [18] using *Delftia* sp. SFG, Nazari et al. [20] using *Serratia marcescens*, Kuroda *et al*. [28] using *Pseudomonas stutzeri* NT-I, Iftikhar et al. [29] using *Bacillus cereus* BTCB 20 and Firouzi Dalvand et al. [19] using *Bacillus licheniformis* PTCC1320.

Growth curve of Bi NPs-synthesizing bacteria and Bi NP production

Fig. 3A shows the absorbance of inoculated media supplemented by Bi^{3+} compared to inoculated culture media without Bi^{3+}. In this study, the amount of Bi^{3+} ions reduction was also demonstrated using the dithizone solution at different times (Fig. 3B). The curve showed that the amount of Bi^{3+} decreases over the time, which indicates the consumption of Bi by bacteria and the production of Bi NP. Furthermore, the most amount of consumption and production of nanoparticles occurred after 24 hours and then in 48 hours (Fig. 3B).

Characterization analysis of biogenic synthesized Bi NPs

The corresponding ultraviolet-visible spectroscopy of the purified Bi NPs are shown in Fig. 3C. Analysis of UV–Vis spectrum shows absorption shoulder at 290 nm, which was similar to previous studies [20, 30].

In the Fig. 3D, in 90% of nanoparticles, the particle size of 22.36 nm is shown in the size distribution pattern of biogenic nanoparticles and has a monomodal distribution graph. The DLS analysis showed a nanoparticle size of 22.36 nm, which appears to be smaller than other Bi NPs previously biosynthesized by other bacteria [18-20, 28, 31-33].

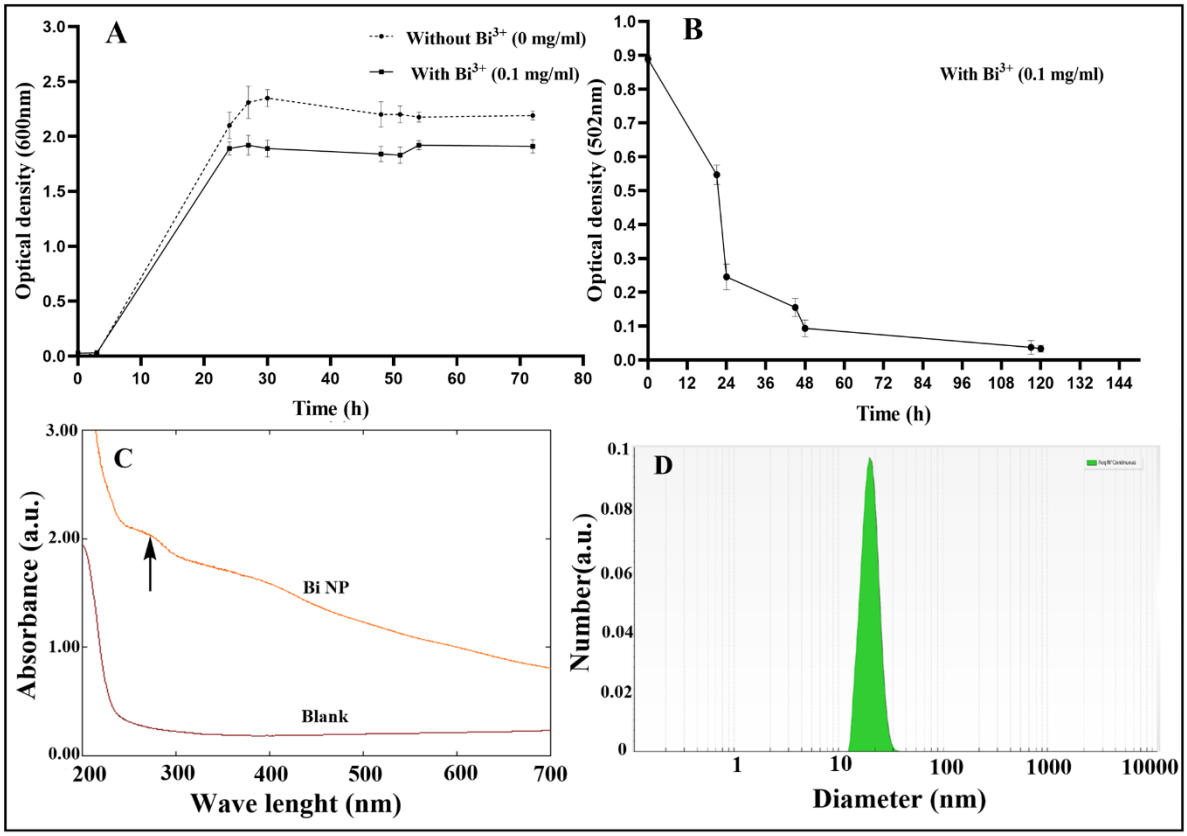

Fig 3. Growth curve of *Bacillus subtilis* strain SFTS in the presence and absence of Bi ions (A), Reduction patterns of Bi ions (B), Ultraviolet-visible absorption ranges of biogenic Bi NPs (C), Size distribution of biogenic Bi NPs (D).

The Bi NPs were also characterized by SEM, EDS, and TEM (Fig. 4). Bi NPs surface morphology was observed near and on the surface of bacteria using SEM. The results showed that the morphology of the synthesized Bi NPs is spherical (Fig. 4A). The EDS results of biogenic synthesized Bi NPs represented signals at 2.2, 2.7, and 8.7 keV as shown in Fig. 4B, which confirms the existence of Bi. Furthermore, the presence of other components is due to the existence of the bacterial culture media. The TEM image also confirms the spherical nanostructures outside and inside of the bacteria (Fig. 4C1 and Fig. 4C2).

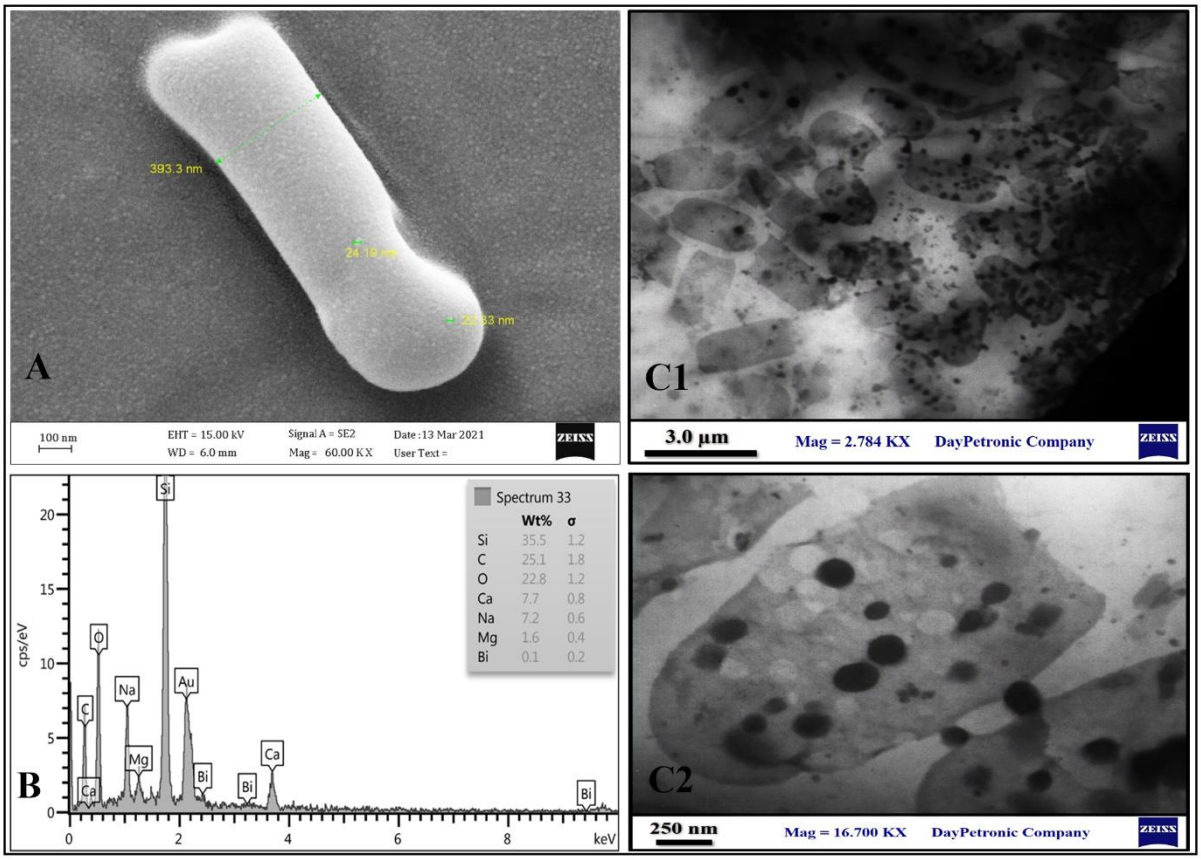

Fig 4. SEM (A), EDS (B) and TEM (C1- C2) images of biogenic Bi NPs.

In Fig. 5(A) the structure and surface chemistry of the Bi NP were characterized by FTIR analysis where the peak values proposed the formation of the nanoparticles. The results of FTIR analysis show the existence of three peaks in areas 3421 cm^{-1}, 1653 cm^{-1}, and 526 cm^{-1}.

In FTIR analysis of Bi NPs, a peak in the region of 3421 cm^{-1} is observed, which can be ascribed to the O-H stretching vibration that mainly for water molecules adsorbed to the surface of nanoparticles. The peak of 1653 cm^{-1} is also can be described as the C=O stretching vibration which might be related to biological active groups on the surface of nanostructures. Also, the absorption band at 526 cm^{-1} originated mainly from the metal-oxygen vibration that represents Bi-O [21].

Fig. 5(B) show the result of XRD analysis, from which it was demonstrated that the biosynthesized nanoparticles by *Bacillus subtilis* strain SFTS consisted only of Bi_4 and monoclinic crystals. In the study by Dalvand et al. [19] where Bi NPs synthesized by *Bacillus licheniformis*, XRD inspection showed Bi_2O_3 with uniform and cubic structure. In another study performed by Nazari et al. [20], Bi NPs synthesized by *Serratia marcescens* represented wide peaks without any clear crystalline structure and the amorphous phase. In addition, Shakibaie et al. [23] who described about Bi NPs synthesis by *Delftia* sp., reported bands associated with the rhombohedral phase from elemental Bi.

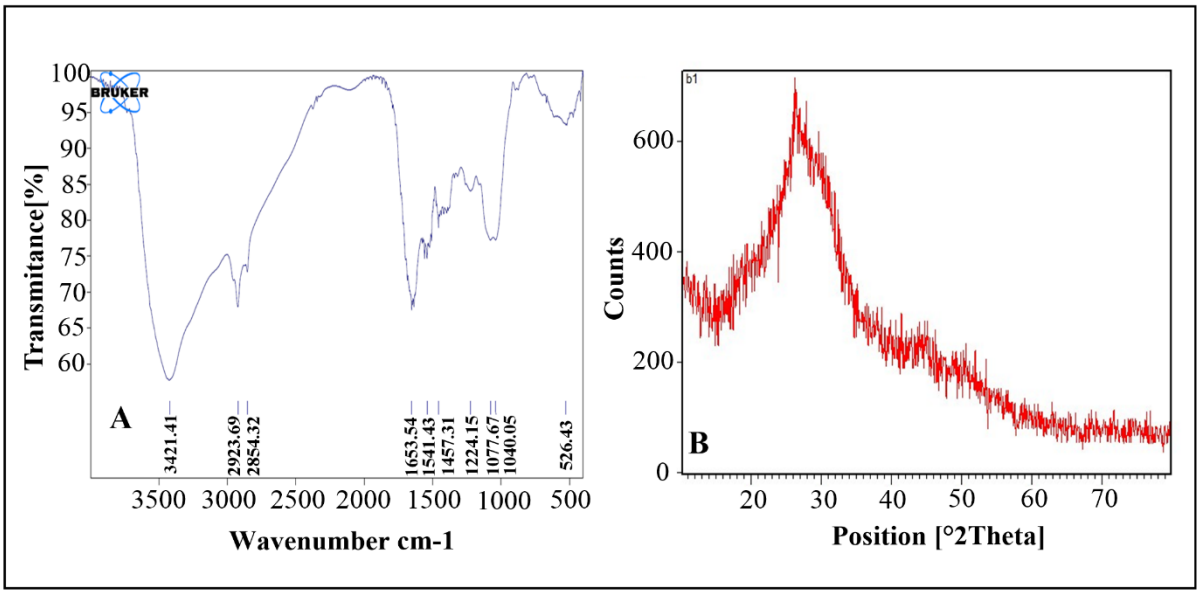

Fig 5. FTIR spectra pattern (A) and XRD profile (B) of Bi NP.

The synergistic effect of Bi NPs in combination with antibiotics

The results of the study of Bi NPs with different antibiotics showed that the nanoparticles have the highest synergistic effect in combination with imipenem and the lowest effect in combination with tetracycline against clinical strains of *E. coli* and *K. pneumoniae* (Table 1). The discs of antibiotics imipenem, ceftazidime, and levofloxacin alone showed a diameter of growth inhibition zone of 22 ± 1 mm, 27 mm, and 9 mm against *E. coli*, and in combination with 40 µl of Bi NPs with a significant difference increased to 27 ± 2 mm, 31 ± 1 mm, and 11 ± 1 mm, respectively (P<0.001). Furthermore, the discs of antibiotics imipenem and levofloxacin alone showed a diameter of growth inhibition zone of 8 mm, 10 mm against *K. pneumoniae*, and in combination with 40 µl of Bi NPs (from stock of 5000 µg/ml) with a significant difference increased to 12 mm and 12 mm, respectively (P<0.001).

Table 1. The evaluation antibacterial activities of Bi NPs alone and in combination with antibiotic discs using disc diffusion assay.

Clinical Bacteria	GIZ [a] of Bi NPs (40 µl / disc from stock of 5000 µg/ml) (mm)	GIZ of antibiotic discs (mm)		GIZ of antibiotic disc + Bi NPs (40 µl / disc from stock of 5000 µg/ml) (mm)
E. coli	8	Imipenem (10 µg)	22±1	27±2
		Ceftazidime (30 µg)	27	31±1
		Levofloxacin (5 µg)	9	11±1
		Tetracycline (30 µg)	R	R
K. pneumoniae	10	Imipenem (10 µg)	8	12
		Ceftazidime (30 µg)	R	R
		Levofloxacin (5 µg)	10	12
		Tetracycline (30 µg)	R	8

[a] Growth Inhibition Zone

In the current study, we analyzed Bi NPs synergistic effect with antibiotics against *E. coli* and *K. pneumoniae*. This examine showed that the highest synergistic effect of biogenic Bi NPs prepared by *Bacillus subtilis* strain SFTS in combination with imipenem and the least

synergistic effect in combination with tetracycline against clinical isolates of *E. coli* and *K. pneumoniae*. Similar results were documented by Rajabi and coworkers who evaluated synergistic effect of Bi NPs in combination with tetracycline and metronidazole on *H. pylori* [12]. Tarjoma et al. [12] showed synergistic effect of the Bi NPs when combined with ciprofloxacin and metronidazole against clinical isolate of *K. pneumonia*. Veloira et al. [13] described about synergistic effect of bismuth thiols particles with ceftazidime, imipenem, and ciprofloxacin on *Pseudomonas aeroginosa* biofilm. The highest synergistic effect was also reported for Bi NPs in combination with ciprofloxacin. In addition, reported the synergistic impact of biogenic Bi NPs synthesized by *Delftia* sp. with cefalexin, ceftriaxone, tobramycin, cefixime, bacitracin, nalidixic acid, and amoxicillin which relatively increased their antimicrobial activity against methicillin-resistant *S. aureus* [18].

Conclusion

In this study, we screened biosynthesized Bi NPs as a biogenic method from the new bacterial isolate, *Bacillus subtilis* SFTS from copper mine soil. TEM images revealed a spherical nanoparticle and the XRD analysis demonstrated that the biosynthesized nanoparticles consisted only of Bi_4 and monoclinic crystals. The results of this study showed that these nanoparticles in combination with various antibiotic discs have a good synergistic effect.

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