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Research Article

Biogenic hydroxyapatite nanoparticle synthesis by *Bacillus subtilis* and its antimicrobial activity.

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ABSTRACT
Hydroxyapatite (HAP) has been widely accepted as a biocompatible ceramic material in several areas of medicine. Several physical and chemical methods are already known for HAP nanoparticles synthesis. These methods are found to be a very expensive and highly toxic. To overcome this issue, the biological methods are found to be a feasible. In current study, bio-inspired hydroxyapatite nanoparticles were synthesized by using bacterial cells of *Bacillus subtilis*. These biogenic nanomaterials were synthesized at room temperature within 2 h. Further, the synthesized nanoparticles were characterized by using Scanning electron microscopy (SEM), Energy dispersive spectroscopy (EDS) and X-Ray diffraction (XRD) techniques. Further, the anti-microbial activity of HAP nanoparticles were tested against the human pathogens, *Salmonella typhi* and *Staphylococcus aureus*. This, study revealed that HAP nanoparticles can be a potential agent in design of an innovative drug against human pathogens.

KEYWORDS
Hydroxyapatite, Nanoparticles, SEM, XRD, Bio-inspired.

1. INTRODUCTION

Recently, nanotechnology has become an emerging field in the area of materials science. A variety of nanomaterials were synthesized which having some novel properties [1]. Such novel nanomaterials have many applications in medicine, environment, cosmetics and technology [2, 3, 4]. Recently, researchers are continuously takes efforts in investigating novel biomaterials with specific applications in field of medicine [5]. It was observed that there is continuous appearance of drug resistance in human pathogens found to be a major challenge for researchers to investigate a novel bioactive materials which having high bio-compatibility and anti-microbial activities against human pathogens [6,7]. Hydroxyapatite (HAP) is the main component of bone and commonly used for reconstruction of the skeleton. They are found to be suitable biomaterials due to its extraordinary biocompatibility, bioactivity, and osteo-conductivity properties. Thus, they have high importance in medical field for implants, coatings, prostheses [8]. The bacterial contamination is common due to their ability to adhere and colonize on surfaces of biomaterials. This is considered to be a serious complication of traumatology and orthopedics [9]. It was seen that the infection rates associated with prosthetic joints are in range of 1-9 % vary from the type of implant [10]. Thus, investigation of HAP nanomaterials with antimicrobial activity is of high importance. The aim of current research work is to synthesized biocompatible HAP nanomaterials via a green route using bacterial cells. Further, need to investigate its antimicrobial activity against the human pathogens.

2. MATERIALS AND METHODS

2.1 Soil sample collection and Isolation of microorganisms

The soil samples were collected from the different garden sites of Waghire College, Saswad, Pune. The soil was samples were collected in sterile plastic bags by using sterile spatula. Further, the collected soil samples were used for isolation of microorganisms. The soil samples were diluted by serial dilution method [11]. After dilution of soil samples, each dilution (0.1ml) was spread on sterile nutrient agar plates. All plates were further incubated at 37^{O}C for 24 h. All experiments were performed in triplicates. The prominent bacterial colony was selected, purified and stored on nutrient agar slants.

2.2 Biochemical and Molecular level characterization of bacterial isolate

The most prominent bacterial colony was selected. Various biochemical tests and 16s RNA technique was carried out for the identification of selected bacterial isolate. The biochemical tests such as Gram staining, oxidase, catalase, fermentation test and IMVIC tests were performed as per previous methodology described [12]. Morphological, cultural, physiological and biochemical properties of the bacterial isolate were studied according to the methods given in Bergey's manual of systematic bacteriology [13].

2.3 Synthesis of Bio-inspired HAP nanoparticles

The prominent bacterial isolate was used for biomass production. The bacterial cell suspension (O.D. 600 = 6.0) was inoculated into 250 ml flasks containing 100 ml nutrient broth. The flasks were incubated for 37^{o}C for 24 h. The grown cells were centrifuged at 10, 000 rpm for 20 minutes. The supernatant was discarded and pellet was collected. The bacterial cell biomass was washed for three times with sterile distilled water and further used for nanoparticle synthesis. For

HAP nanoparticle synthesis, a bacterial cell biomass standard suspension was prepared by adding 1:1 ratio of bacterial cells and sterile distilled water. 25 ml of cell biomass standard suspension was added into 25 ml of aqueous solution of 1.0 M calcium chloride and vigorously stirred at room temperature. Further, a solution of 0.6 M phosphoric acid (25 ml) was added slowly in drop wise manner to the vigorously stirred calcium chloride solution. Resultant precipitation was collected and thoroughly washed for several times with sterile distilled water and further air dried.

2.4 Characterization of HAP nanomaterials

The synthesized HAP nanoparticles were characterized. Scanning electron microscopy (SEM) and elemental analysis of HAP were carried out on platinum coated HAP samples that were properly air dried on silicon wafers. An analytical scanning electron microscope attached with energy dispersive spectrometer (EDS) was used. For X-Ray diffraction (XRD) of HAP was performed by forming dried thin films of HAP nanoparticles on silicon wafers described earlier [7].

2.5 Antimicrobial activity of HAP nanoparticles

Antimicrobial activity of HAP nanoparticles were tested against human pathogens like *Salmonella typhi* and *Staphylococcus aureus* by agar diffusion technique. In this method, 0.1ml of bacterial cell suspension was spread on sterile nutrient agar plates. Further, wells were prepared on nutrient agar plates. About, 40 µl of synthesized nanoparticles were added into each well except one which was kept as control. All plates were kept for diffusion at 4ºC for 30 minutes. Further, all plates were incubated at 37ºC for 24 h. After incubation, the zome of inhibition was observed [7].

3. RESULTS AND DISCUSSION

3.1 Isolation, characterization of soil isolate and synthesis of HAP nanoparticles

The prominent bacterial isolate was studied for their colony characteristics on nutrient agar plate and Gram nature of bacterial isolate under light microscope as shown in figure 1.

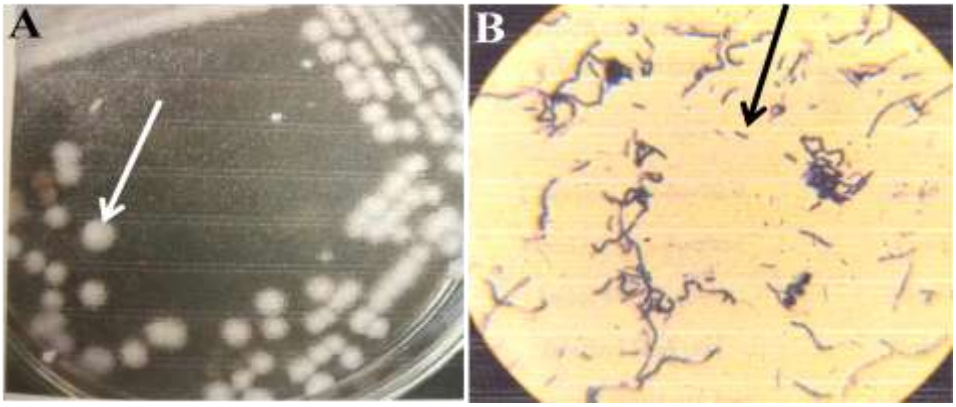

Figure 1. (A) Colony morphology of bacterial isolate and (B) Gram staining observation under light microscope.

Table 1. Colony characterization of bacterial isolate

Basic characteristics	Properties
Size	4 mm
Shape	Circular
Colour	Milky
Margin	Regular
Opacity	Opaque
Elevation	Convex
Consistency	Non sticky
Gram staining	Gram positive
Motility	Non motile
Shape of vegetative cells	Rod

Table 1 reflects the colony characterized observed on nutrient agar plate. Figure 1 shows the isolation of soil bacteria having milky colour (Fig. 1A) and shows violet coloured rod after performing Gram staining indicates the isolated bacteria is Gram positive in nature (Fig. 1B).

Table 2. Biochemical test and identification of soil isolate.

Biochemical and growth characteristics	Properties
Catalase	Positive
Nitrate reductase	Positive
Oxidase	Positive
Voges-proskaeur	Negative
Indol	Positive
Methyl red test	Negative
Voges-proskaeur	Positive
Citrate	Positive
Aerobic growth	Positive
Anaerobic growth	Negative
Optimum temperature	37ºC
Optimum pH	7.0
Glucose, Lactose, Maltose and Sucrose	Acid production without gas

The table 2 reflected the biochemical and cultural characteristics of soil isolate. It was seen that based on biochemical characteristics, the soil isolate belongs to *Bacillus* spp. The obtained results were in a good agreement with the previous report [14]. Based on 16 sRNA DNA sequencing it was identified as *Bacillus subtilis* AB02. The partial sequence was deposited to NCBI (http://www.ncbi.nlm.nih.gov/nuccore/KX516728) and obtained accession no KX516728.

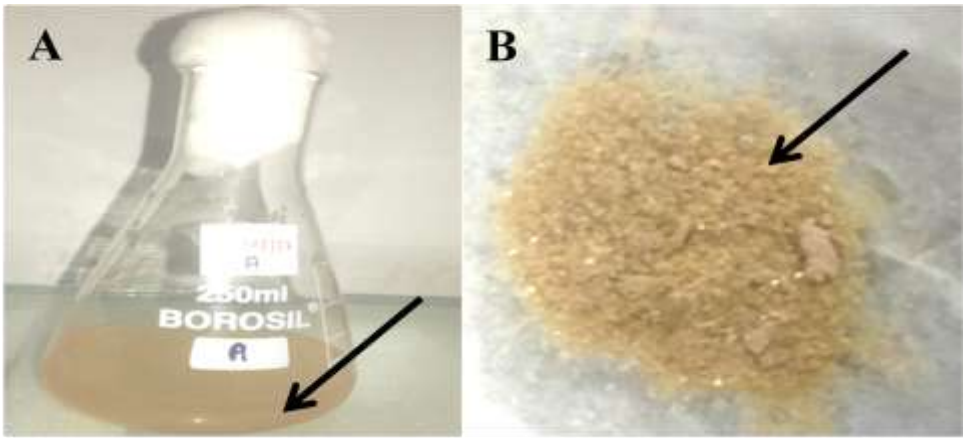

Figure 2 (A). Biomass production of *Bacillus* Spp in nutrient broth (B) HAP nanoparticles synthesized by *Bacillus* Spp.

Figure 2 reflects that biomass of *Bacillus* Spp was produced (fig 2A) and biomass was utilized for HAP nanoparticles synthesis (fig 2B). In similar manner, HAP nanoparticles were also synthesized by co-precipitation method is reported earlier [9]. Thus, current method is found to be a simple, eco-friendly and cost effective.

3.2 Characterization of HAP nanoparticles

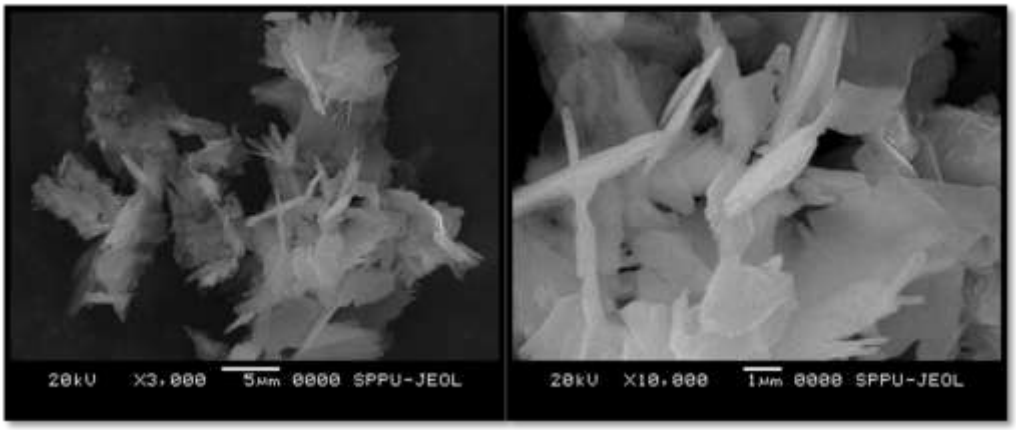

Figure 3. SEM images of HAP nanoparticles synthesized by Bacillus spp.

Figure 3 reflects that HAP nanoparticles are assembled together and formed butterfly like structures. Similar HAP nanoparticles assembly was observed when they were synthesized by co-precipitation method reported previously [9]. The elemental composition was analyzed by using EDS as shown in figure 4.

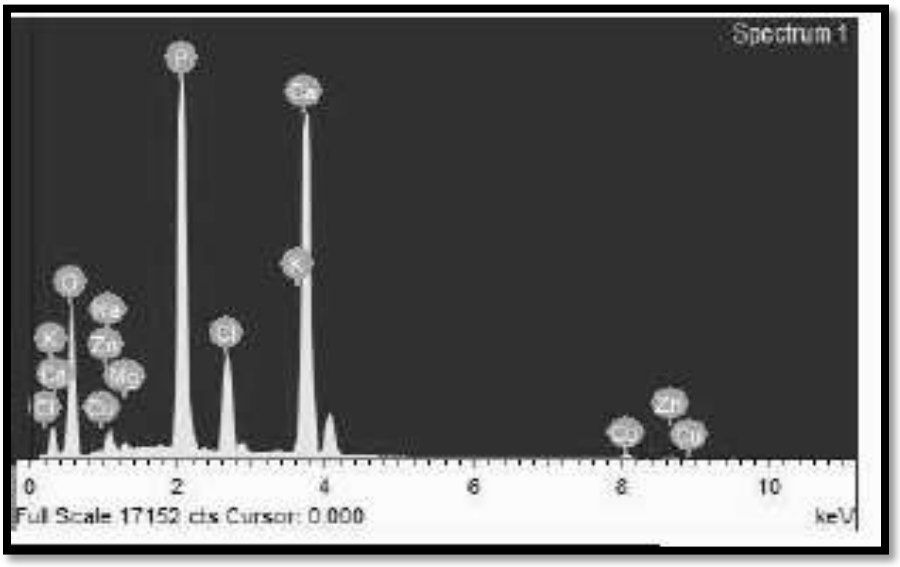

Figure 4. The Spot-EDS profile of HAP nanoparticles synthesized by *Bacillus* spp.

Figure 4 revealed the presence of major peaks of Ca and P in indicates their presence in the hydroxyapetite crystalline structures. Thus, it was confirmed that the structures are observed in SEM images (fig. 3) are HAP nanomaterials.

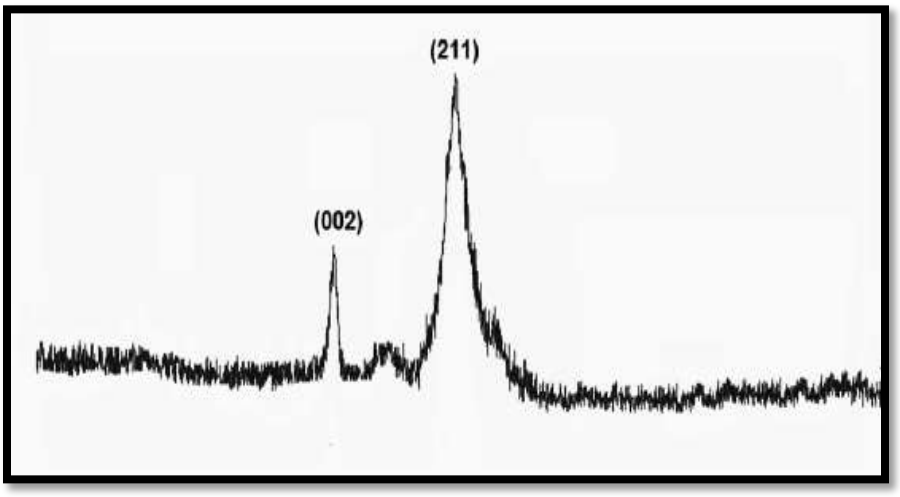

Figure 5. The representative XRD profile of HAP nanoparticles synthesized by *Bacillus* spp.

The synthesized HAP nanoparticles were further characterized by using XRD. The XRD patterns revealed the crystalline phase of hydroxyapatite nanoparticles. Figure 5 of XRD reflected the characteristics of the (002) and (211) planes. The obtained results are in good agreement with previous report [15]. Thus, the XRD profile revealed the crystalline nature of HAP nanoparticles synthesized by *Bacillus* Spp.

3.3 Antimicrobial activity of HAP nanomaterials

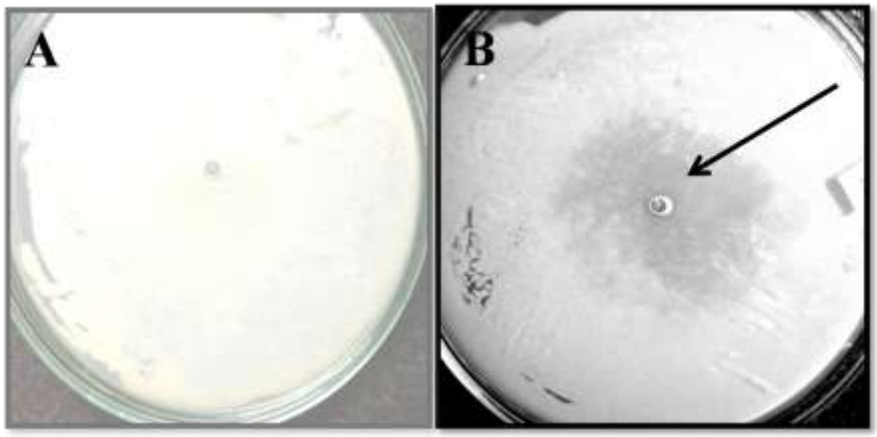

Figure 6 A). Control- No zone of inhibition B) Zone of inhibition was observed by HAP nanoparticles against *S. typhi.*

Figure 6 revealed that HAP nanoparticles synthesized by *Bacillus* Spp have antimicrobial activity against *S. typhi and Staph. Aureus*. Such activity was also observed by Ag:HAP nanoparticles when tested against *Staph aureus*, *Klebsiella pneumoniae*, *Providencia stuartii*, *Citrobacter freundii* and *Serratia marcescens* [9]. Thus, anti-microbial activity associated with HAP nanoparticles can be additional benefit when they will be used in transplant purpose.

4. CONCLUSION

In current study, bio-inspired HAP nanoparticles were synthesized by using green method in which *Bacillus* Spp was used. The synthesized HAP nanoparticles were challenged for their antimicrobial activity against the human pathogens such as *S. typhi* and *Staph aureus*. This study revealed that HAP nanoparticles can be an effective agent against the human pathogens and can be useful as biocompatible material in design of innovative drug against the human pathogens in future.

5. ACKNOWLEDGEMENT

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