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BIOSYNTHESIS OF MAGNETIC IRON OXIDE NANOPARTICLES FROM *BACILLUS SUBTILIS* STRAINS: AN IN-VITRO ESTIMATION OF ITS ANTIBACTERIAL AND ANTIOXIDANT PROPERTIES

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Abstract: Nanoparticles have been revolutionary in modern biology for many reasons. Different kinds of nanoparticles are catered for different uses, one of them being magnetic in nature. Out of many methods of synthesis of Magnetic nanoparticles (MNPs), microbial synthesis is safer and more cost-effective. Ferrite nanoparticles of size 128 nm are the most explored MNPs so far. They can be successfully implemented in wastewater treatment, cancer diagnosis and treatment. This review discusses the methods to prepare nanoparticles of iron nature and test their magnetic, antibacterial and antioxidant activities. Bacillus subtilis strains were isolated from soil by serial dilution technique then cultured in a specific nutrient medium to allow the growth of magnetic bacterial strains. Once magnetic bacterial strains are confirmed, they are centrifuged, diluted, and finally tested by a magnet to analyze the attraction of synthesized MNPs. Furthermore, the synthesized MNPs are examined for antimicrobial activities against selective bacteria and especially antioxidant activities.

***Index Terms - Bacillus subtilis*, magnetic nanoparticles, ferrite nanoparticles, antibacterial**

I. INTRODUCTION

In recent times, nanoparticles have had a wide range of applications in biological studies[11]. The size of nanoparticles is the most attractive feature that makes them valuable in innovation. Current studies are attempting to perfect the use of nanoparticles for drug delivery, therapeutics, neuroscience and antimicrobial applications. Nanoparticles have been derived from various sources and, in this case, bacterial species prove to be significant. Microorganisms, showing a great deal of biodiversity, provide a range of options to manufacture MNPs and this can be done in large quantities[10]. Furthermore, the discovery of magnetotactic bacteria by Salvatore Bellini in 1963 has led to its uses in geology, the food industry and cell separation techniques[1]. In comparison to other nanoparticles, magnetic properties, low toxicity, high bio-compatibility and easy synthesis favours the use of iron oxide nanoparticles in pharmaceutical and drug-related research. Exploitation of iron oxide nanoparticles (IONPs) helps to enhance magnetic hyperthermia treatment. DNA extraction is also made possible by coating IONPs with material such as silica or chitosan that acts as an adsorbent.

In this study, *Bacillus subtilis* strains isolated from a soil sample are utilized. Iron is a very important source for the growth of bacteria but its availability in the soil is dictated by environmental factors such as pH and oxygen content of soil. Iron, in ferric form, is absorbed from the soil by siderophores produced by *Bacillus subtilis*. The concentration of iron influences the formation and regulation of biofilm which is essential to bacteria for protection against calamities and absorption of organic compounds[2].

A simple nutrient enrichment medium has been prepared and used to support the development of iron oxide nanoparticles in the bacterium species during incubation[1]. Properties such as magnetic nature, antimicrobial and antioxidant activities are tested for the synthesized IONPs. Previous studies have shown that iron oxide nanoparticles display "excellent" antimicrobial and antioxidant activities in other bacterial and fungal species. Also, ferrite nanoparticles are the most studied magnetic nanoparticles. Another objective of the study is to confirm the effectiveness of iron oxide nanoparticles from *Bacillus subtilis*. The sample is taken in small quantities to minimize errors and wastage of materials.

II. MATERIALS AND METHODS

2.1 Materials

Nutrient agar, Luria-Bertani broth, 9K media, 0.9 M NaCl, 0.2% sodium dodecyl sulphate, 0.1 mM 2,2-diphenyl picrylhydrazyl

2.2 Extraction and culture of bacterial sample

Bacillus subtilis is isolated from the soil sample using the serial dilution technique and the spread plate method.

2 test tubes are taken for serial dilution with 2.5 mL distilled water in test tube I and 2 mL distilled water in test tube II. 0.1g soil is weighed and added to tube I. The tube contents are vortexed and 500 µL is extracted and added to tube 2.

Streaking plate method used to get single colonies of pure culture. The contents from tube 2 are streaked on nutrient agar plate and the plate is incubated at 37° C overnight.

2.3 Culture in LB and 9K media

Bacterial isolates are then cultured in both Luria Bertani broth and 9K medium. This is done to check for the growth of magnetotactic bacteria[1].

2.4 Biochemical confirmatory tests and Gram staining

The bacterial cells are subjected to Gram staining. To confirm if the species is Bacillus, biochemical tests such as the catalase test, starch hydrolysis test, carbohydrate fermentation test, methyl red test, Vogue-Proskauer test and citrate test are performed in vitro[8].

2.5 Detection of magnetic movement

2 mL bacteria are cultured in 9K medium following incubation time of 24-48 hours. This is collected twice in 2 Eppendorf tubes which are spun at 6,000 g for 10 minutes. Pellets are obtained which are washed thrice with 0.9M NaCl. After washing, the pellets are resuspended in distilled water. Pellets are tested for magnetic activity by using an external magnet and are re-cultured in 9K medium[1].

To validate this, Bacillus subtilis cells are grown in LB media. Cells are spun at 3000 g and the supernatant is mixed with 9K media and incubated. LB containing debris is also mixed with 9K media and this mixture is also tested for presence of magnetic bacterial strains[1].

2.6 Magnetosome extraction

After incubation period, magnetic bacterial strains are collected in Eppendorf tubes and centrifuged at 13,000 g for 10 minutes. Following centrifugation, pellets obtained are washed thrice with 0.9 M NaCl. Then, they are suspended in 0.2% sodium dodecyl sulphate and incubated at room temperature for 45 minutes[1]. After incubation, extracted magnetosomes are washed with 0.9 M NaCl and stored in 1 mL PBS. Magnetic activity is checked again using an external magnet[1].

2.7 UV-Vis spectrophotometry

Suspension of the iron oxide nanoparticles is scanned with the UV-Vis spectrophotometer. OD is recorded against spectra ranging from 200 to 600 nm. Distilled water is taken as blank[9].

2.8 Antibacterial activity

The well diffusion method is used to evaluate the antibacterial activity of magnetic IONPs against S. aureus [Gram positive] and

E. coli [Gram negative] [4]. 200 µL of the bacterial suspension is spread on agar medium and streaked. Four wells are made on agar medium for distilled water, antibiotic chloramphenicol, 50 µL and 100 µL iron oxide nanoparticle solution. Each of these are added in defined volumes inside the wells. The agar plates are kept for incubation. After incubation, the measurement of the inhibition zone was recorded in both cases to indicate the antibacterial activity.

2.9 Antioxidant activity

The 2,2-diphenyl picrylhydrazyl (DPPH) scavenging assay is employed to determine the antioxidant activity of the iron oxide nanoparticles. 2 test tubes are taken as blank and sample. 0.2 mL of IONPs was mixed well with 1 mL of freshly prepared 0.1 mM DPPH in each test tubes. The resulting solution was incubated in the darkroom for 30 min. After incubation, the absorbance of the mixture solution was recorded at 517 nm using UV-spectrophotometer[7]. The % DPPH value was calculated from the OD

values.
Formula used for calculation:

$$\frac{\text{Absorbance[Blank]} - \text{Absorbance[Sample/Standard]}}{\text{Absorbance[Blank]}} \times 100$$

III. RESULTS AND DISCUSSION

3.1 Identification and characterization of *Bacillus subtilis*

Table 1: Biochemical confirmatory tests for *Bacillus subtilis*

SL.NO	BIOCHEMICAL CONFIRMATORY TESTS	RESULT
1.	Catalase test	+
2.	Starch Hydrolysis	+
3.	Citrate	+
4.	Carbohydrate Fermentation test i) Glucose ii) Maltose	 + +
5.	Methyl red test	-
6.	Vogue Proskauer test	+
7.	Endospore staining	+

Bacillus subtilis is notable for its fast growth, rod-shaped cells, motility and Gram-positive nature. Cells were streaked hexagonally and are visible on the nutrient plate. This was kept for incubation for 24 hours overnight. Microscopic images prove the bacterial structure. Bacterial cells cultured on nutrient agar plate are isolated and re-cultured in LB media and 9K media to obtain pure isolates. These isolates are further used for testing in the experiment. A series of in-vitro tests help to confirm the bacterial species.

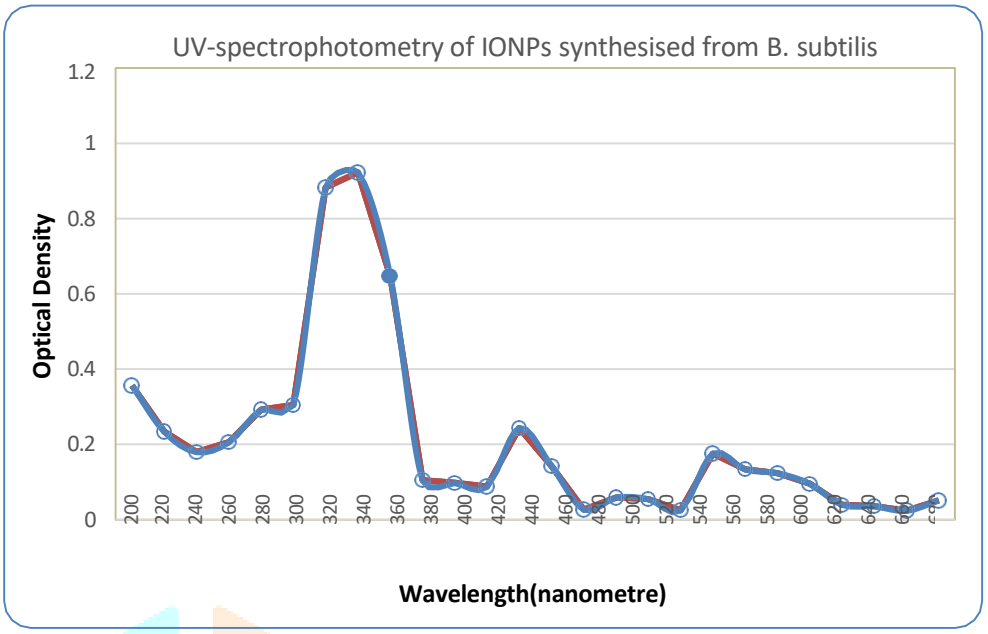
3.2 Magnetic nature

After 9K medium inoculated with isolates of *Bacillus subtilis* has been incubated overnight (24 hours), the bacterial cells assumed magnetic nature. After isolation into tubes and centrifugation, an external magnet was placed near one end of the tube. The bacterial cells slowly aligned in a parallel direction facing the magnet. At the end of the experiment, the newly synthesized magnetosomes similarly displayed magnetic behaviour[1].
No magnetic movement occurs in bacterial cells cultured in LB medium, confirming that 9K medium is responsible for the biosynthesis of magnetic bacterial strains.

3.3 UV-visible spectrophotometry

The ultraviolet spectrum was measured at a range of 200-700 nm. The appearance of an absorption peak at a wavelength of 340 nm is an indication that the iron oxide nanoparticles (IONPs) have formed. The aggregation state of the nanoparticles and the environment surrounding them affect the UV-visible absorbance spectrum. A wide absorption peak indicates that there is aggregation among the IONPs.

Graph 1: UV-Spectrophotometry of Iron oxide nanoparticles



3.4 Antibacterial activity

Table 2: Antibacterial activity of the synthesized iron oxide nanoparticles by Bacillus subtilis against Gram- negative and Gram-positive bacteria.

Cell	Diameter of inhibition zones (cm)			
Bacteria/ IONPs conc. (µL)	**Distilled water (µL)**	**Antibiotic (µL)**	**IONPs (50 µL)**	**IONPs (100 µL)**
E. coli	ND	3.5	0.5	1.7
Staphylococcus aureus	ND	3.5	ND	ND

The well diffusion method estimates the antibacterial activity of magnetic Iron oxide nanoparticles against S. aureus and E. coli. From the tabular values, it is inferred that IONPs show more pronounced bacteriostatic actions against Gram-negative *Escherichia coli* in comparison to Gram-positive *Staphylococcus aureus*. Antibiotic Chloramphenicol was used and showed similar zones of inhibition at 3.5 cm in both cases. It is inferred that 100 µg of IONPs exhibited better antibacterial activity against E coli with the zone of inhibition measured at 1.7 cm in comparison to the zone of inhibition formed by the addition of 50 µg of IONPs at 0.5 cm.

3.5 Antioxidant activity

Table 3: Antioxidant activity of the synthesized iron oxide nanoparticles by Bacillus subtilis

Sample	OD values	% DPPH
Blank (distilled water)	**1.55**	-
IONPs	**1.340**	**13.54**

The DPPH assay is very quick and efficient to be employed in the case of measuring antioxidant activity. UV-vis spectrophotometry technique is again utilized to check this activity and record the values.
Iron oxide NPs show appreciable antioxidant activity at 510 nm using DPPH method. A lower DPPH percentage indicates better scavenging activity of antioxidant samples[7]. The iron oxide nanoparticles

showed the potent DPPH radical scavenging value at 13%.

IV. CONCLUSION

The biosynthesis of magnetosomes is very cost-effective and efficient. IONPs have been successfully synthesized from Bacillus subtilis in small quantities and also displays moderate magnetic behaviour. It also shows appreciable antibacterial and antioxidant activities. Although it isn't well effective against Gram positive bacteria, more intensive research could be done on improving it. Concentration exceeding 100 µL of magnetosome can be added for better results of antibacterial activity against Gram-positive bacteria[5]. Harvesting bacteria in larger quantities of nutrient media could yield better metallic nanoparticles.

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