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Original Research

Biosynthesis and characterization with antimicrobial activity of TiO2 nanoparticles using probiotic *Bifidobacterium bifidum*

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Abstract: *Bifidobacterium* selectively colonizes the infants' intestinal tract, and the relevant coliform bacteria in adults are particularly beneficial because of their enhanced capability to prevent pathogens of gastro intestine by direct antimicrobial action and relieve infection, which led to their intensification, the antibacterial activities of titanium nanoparticles producing by some bacteria, makes them attractive as a new agent against pathogenic bacteria. In our present study, we used a probiotic bacteria *Bifidobacterium bifidum* which was isolated from the commercial market capsule to produce TiO2 nanoparticles and study the biologically characterized nanoparticle using various techniques like Scanning Electron Microscopic (SEM), atomic force microscopy (AFM), and study its antimicrobial activity against a bacteria isolated from the stool of patients suffering from acute diarrhea. The results showed that the morphological characteristics of nanoparticles were found to have a spherical shape and mean size of 81 nm by AFM while scanning electron microscope viewed as an oval shape with anatase form synthesized by *B. bifidum*. TiO2-NP synthesized by *B. bifidum* had an inhibitory effect against *P. aeruginosa, A. baumanii, K. pneumonia* at a concentration 16 mg/ml and 32 mg/ml towards *E. coli and S. typhi*, the minimum inhibitory concentration (MIC) against pathogenic bacteria isolated from acute diarrhea included *Pseudomonas aeruginosa, Acinetobacter baumanii, Klebsiella pneumonia, E.coli and salmonella typhi* was utilized to determine the antibacterial impact of the synthesized TIO2 nanoparticles. Our biologically synthesized titanium nanoparticles were effective against all the tested pathogenic bacteria at various degrees and had a probable role in significantly greater antimicrobial efficacy against all isolates under study. This trial may have considerable significance for the prevention of antibiotic resistance associated diarrhea in hospitals.

Key words: *Bifidobacterium bifidum*; TiO_2 nanoparticles; Acute diarrhea.

Introduction

As living microorganisms, probiotics supply health advantage to the host when ingested in sufficient amounts (1). Probiotic bacteria are believed to show an important role in the digestive system and therefore must survive the passage from the stomach into the intestine (2).

Bifidobacteria is the dominant microorganism in the human digestive tract of adults and infants focused on the prevention of diarrhea and allergies (3). There are many health benefits associated with Bifidobacteria, and the resulting commercial significance resulting from their incorporation into functional foods (4). Oxidative stress can severely affect the viability of Bifidobacterium exposure of *Bifidobacterium* cells to oxygen causes the accumulation of reactive types of oxygen, especially hydrogen peroxide, leading to cell death (5).

Because of the remarkable chemical and physical properties of nanomaterials, nanomaterials such as TiO 2 nanoparticles (TiO 2 -NPs), with a diameter of less than 100 nm, have become a new generation of advanced materials thanks to their brilliantness as well as interesting optical, dielectric and photo-catalytic characteristics from size quantization, resistant strains do not grow if nanoparticular formulations are applied in their media. Many nanoparticles ' antibacterial activities make them attractive in their function as new agents against pathogenic bacteria (6).

The microorganisms are employed as probable nanofactories to develop environmentally friendly, nontoxic, and clean approaches for fabricating nanoparticles (7). Research has focused on numerous probable antibacterial agents such as metal oxides with nanometer size. Since it has been found that some such agents are cytotoxic against bacteria but not against mammalian cells, it is possible to use them for medical purposes (8). The current study was carried out to fulfill the following objectives:

1. Isolated *Bifidobacterium bifidum* from the commercial market capsule to produce tio2
2. Nanoparticles biologically with studying their effect against pathogenic bacteria isolated from acute diarrhea.
3. Investigate the characteristics of titanium nanoparticles by Atomic Force Microscopy and Scanning Electron Microscope.

Materials and Methods

Samples collection

Samples were collected from patients with diarrhea who had known clinical symptoms. Samples were collected from Razkari Teaching Hospital.

Isolation and identification of bacteria causing diarrhea

Bacteria were isolated as pure colonies on McConkey and blood agar. Therefore, the bacteria were microscopically checked using the Gram stain method showed as Gram-negative bacteria. Tests of Identification included cultural, morphological, and physiology characters in each bacterial isolation. Bacterial isolate colonies grew on blood and McConkey agar was defined in terms of their form, colour, diameter, odor and other characteristics (McFaddin.2000) (9) Isolated and identified during another study (data not shown).

Identification of Gram-negative bacteria causing diarrhea by using Vitek 2 compact system

Streak the surface of McConkey agar to be diagnosed with isolated bacteria and incubated at 37C^{o} for 24-48 hours. The GN and GP cards were removed from the cover, and the model number for the unit was registered. A sufficient number of pure colonies was suspended in a 3 ml of physiological saline solution in a transparent plastic test tubes. The isolated bacterial suspension was measured to be diagnosed by the turbidity device Vitek2 (Densichek) which the turbidity must equal to (0.50-0.63). The card (tube unit) transferred which is on stand transfer the stand the vacuum chamber in the device where the card was inoculated.

A proper inoculation survey internally was conducted, then the transport tube of the equipment was cut off, and then transferred it to the incubator card, insulated at 35 ^{o}C, and read the results within 8 hours or less, and then during the incubation period analyzed the equipment. Biochemical and storage mode (biochemical mode) was subjective, and there was a printed diagnostic report for each card in the reader/incubator according to the company biomerieux instructions.

Cultivation of Bifidobacterium bifidum

One gram of commercial product was dissolved a sterile saline solution (9 ml). Then, 1 ml of the obtained suspension was cultured on in the De Man, Rogosa, Sharpe MRS broth under anaerobic condition (Gaspack H_2+CO_2), all media were supplemented with 0.3 g/l L-cysteine-HCLand 02% (wt/ vol) Na2C03 and 01 % (wt/vol) CaCI2·2H20. Active cultures were incubated aerobically for 48 h at 37 °C and subcultured at least twice before the experiment.

Biosynthesis of nanoparticles using *Bifidobacterium bifidum*

A flask containing 100 ml MRS broth was inoculated with 2% of the fresh culture of *B. bifidum*. The flasks were incubated at 37 ^{o}C for 24 h. cultures were centrifuged at 12,000 rpm for 5 min, their supernatants were filtered by Millipore filter paper (0.22 µm), then 20 ml of supernatants were added to a flask containing 10 ml of TiO2 solution and was stirred for 30min using magnetic stirrer then incubated at 37 ^{o}C for 48 h. There was a change in the color, and TiO2 nanoparticle production was detected by observing sediment production (10).

Detection for TiO_2 nanoparticles synthesis

Three flasks were used, each flask filled out with 40 ml of MRS broth. And 20 ml of TiO_2 (0.025m) to the first and second flasks were added respectively, and both were stirred on a magnetic stirrer for half an hour while the third flask held only MRS broth. In the end, the final concentration would be equal. *B. bifidum* isolates were cultured in first and third flask incubated anaerobically at 37 ^{o}C for (24, 48, 72) hours. The second flask was used as blank for the first one, the color change from light brown to dark brown is observed and sediment production is observed as the primary detection of the created TiO_2 nanoparticles (11).

Characterization of biosynthesized nanoparticles

Samples of biosynthesized nanoparticles (prepared by drying sediment at room temperature) were characterized after 72 h of incubation. Scanning Electron Microscopic (SEM) and Atomic Force Microscopy (AFM) were used to confirm the formation of metal oxide TiO2 nanoparticles, the images of NPs were achieved in a Scanning and Atomic electron microscope at Alrazi Metallurgical Research Center, Tehran, Iran.

Characterization of titanium nanoparticles by Atomic Force Microscopy

Atomic Force Microscopy image was obtained using AFM XE 100 Park system. The aqueous titanium nanoparticles were deposited on a substratum of freshly cleaved mica. The sample aliquot was left for 1 min, washed with deionized water then left for 15 min to dry. The photos were obtained by scanning the mica in the air in non-mode of touch (12). The size, shape, and disparity mode of TiO_2 nanoparticles were determined by Atomic Force Microscopy.

Characterization of titanium nanoparticles by Scanning Electron Microscope

To know structure, shape, thin film of the titanium nanoparticle powder was made on an adequate aluminum plate by just dropping a very small level of the sample on the plate, an extra solution was removed using a blotting paper and the film was then allowed to dry overnight on the plate. The SEM analysis was done instrument operated at an accelerating voltage of 15.00 kV (13).

Stability of synthesized TiO_2nanoparticles

The flask containing 100 ml sterilized MRS broth was inoculated (2 ml)with a fresh culture of *B. bifidum.* The culture was incubated at 37C^{o} for 72 hours. After incubation the culture flasks were kept at 4 C^{0} for 3 months, then the stability of color and aggregation of nanoparticles in media was optically observed.

Antibacterial activity of nanoparticles

Agar Well diffusion- method

Titanium TiO2 nanoparticles produced by *B. bifidum* were screened for their inhibitory activities against

acute diarrhea pathogens under our study using agar well diffusion method. The plates were made ready by dispersing about 105 cfu/ml bacterial indicator broth culture of isolate on the nutrient agar surface. The plates then left for 15 min before adding the 50µl of TiO2-NPS synthesized by *Bifidobacterium bifidum* for each concentration (2000, 200, 100) mg/ml in to the agar wells were bored in the agar plates before. Afterward, incubation of the plates was performed at 37°C for 24 h. Zones of inhibition were calculated.

MIC Method

Minimum inhibitory concentration (MIC) rates are referred to as the minor concentration level of TI-NPS at which growth does not visibly happen following the incubation for the target time. MIC is used to determine the antibacterial activity of TiO2 -NPS. As described by Morello *et al.* (2003) (14). broth dilution method was employed to determine MIC towards *Pseudomonas aeruginosa, Acinetobacter baumanii, Klebsiella pneumoni., E. coli and Salmonella typhi*. A stock solution of TiO2 NPS from *B. bifidum* was diluted in sterilized distilled water to concentrations including 4, 8, 16, 32, 64, and 128 mg/ml.

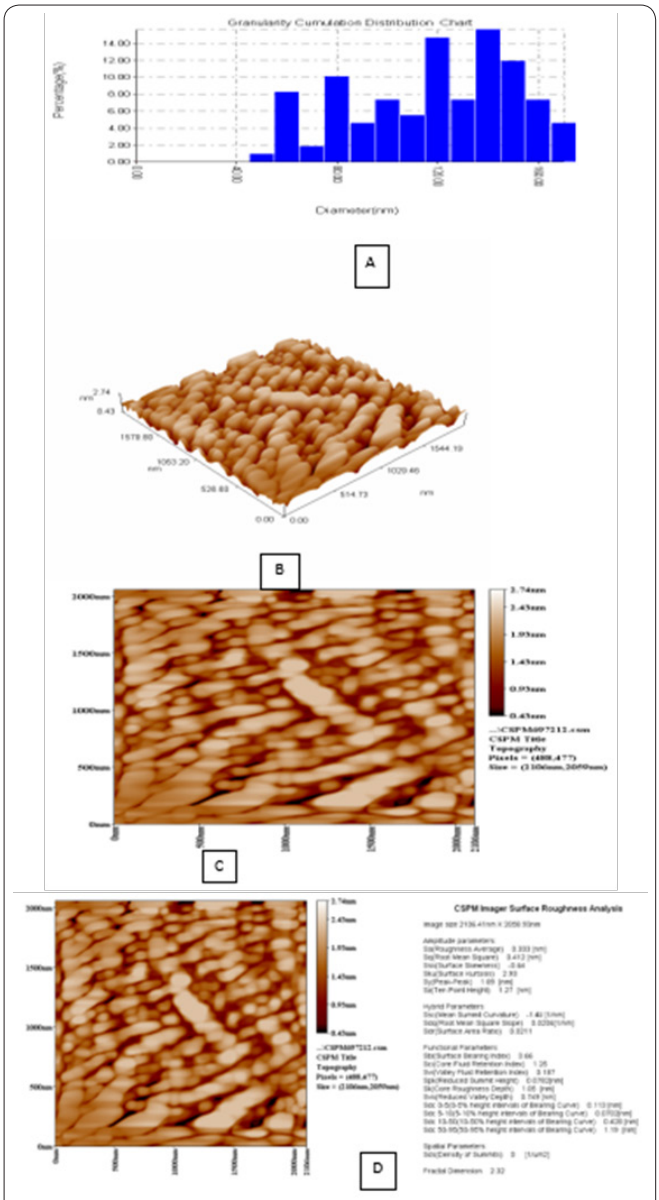

Figure 1. The image obtained from Atomic Force Microscopy for TiO2 nanoparticles synthesized by *Bifidobacterium bifidum***.** A: Diameter percentage, (B, C, D): Surface and three dimensional view.

Results

The present study involved the synthesis of various-size nanoparticlesTiO2NPs by bacteria *Bifidobacterium bifidum* which were characterized regarding their surface charges, dimensions, and morphologies. This study covered particle size was analyzed by Atomic Force Microscopy. Surface and 3D views of the nanoparticles were obtained by utilizing AFM, and it was found that the average size of particles was 18 (Figure 1), while scanning electron microscope viewed as an oval shape with anatase form Synthesized by *B. bifidum* (Figure 2).

Antimicrobial activity of TiO2 NPS from bifidobacteria was examined and a well diffusion method using different concentrations (2000, 200, 100) mg was employed to determine the antibacterial activity of TiO2 NPS separated from *Bifidobacterium bifidum*. The diameter of inhibition zones around each well represented in (Table 1). showed maximum zone of inhibition against *S. typhi, A. baumani, E. coli* and *P. aeruginosa* 28 mm and *K. pneumonia* 26 mm at 200mg and the least inhibition zone was observed against *P. aeruginosa* (18 mm), while equal inhibition zone showed by *A. baumani* and *K. peumonia* 20 mm followed by *E. coli* and *S. typhi* 22 mm at 100 mg concentration (Table 1).

MICs are most widely used as a study tool to assess new antimicrobial *in vitro* activity, for each bacterium, the MIC was specified (Table 2). It was found that the MIC of TI-NPS against *S. typhi* and *E. coli* was 32mg/ml, while the MIC *for Pseudomonas aeruginosa, A. baumanii, K. pneumonia* isolates were noticed at 16 mg/ml MIC. Variations in the tested genus and species

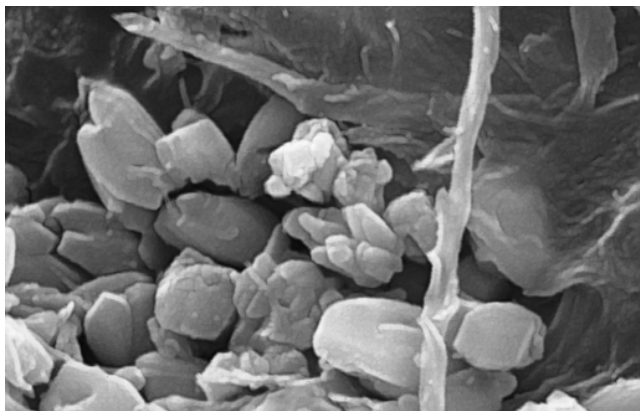

Figure 2. Images obtained from scanning electron microscopic for TiO_2 nanoparticles synthesized by *Bifidobacterium bifidum*.

Table1. Inhibition zone of TiO_2 nanoparticles synthesized by *B. bifidum.*

Bacterial isolates	Inhibition zone (mm)		
	2000 mg	200 mg	100 mg
P.aeruginosa	36	28	18
E.coli	33	28	22
A. baumani	28	28	20
K.pneumoniae	23	26	20
S.typhi	26	28	22

Table 2. MIC (mg/ml) of TiO_2 nanoparticles and bacterial isolates.

MIC (mg/ml)				
S.typhi	***K.pneumonae***	***E. coli***	***P.aeruginosa***	***A.baumannii***
32	16	32	16	16

possibly lead to varieties in MIC of TiO_2 nanoparticles (Table 2).

Discussion

Bifidobacterium is a genus of Gram-positive, non-flagellated and often rod-shaped anaerobic bacteria, colonies forming on agar plates are spherical, convex, creamy or white, shiny, smooth, neat-edged, sticky, most strains cannot expand below 90% air and 10% CO2. It was necessary to examine each isolated colony by light microscopy to differentiate bifidobacteria from other colonies. The selective medium used is particularly suited for promoting the typical Y type of bifidobacteria (15).

A new feature of probiotics is discovered every day, and new uses are established for them. One of these new aspects is the forming of nanoparticles by *B. bifidum* isolate so our trail concentrated on that *Bifidobacterium bifidum* was examined for the production of TiO2NPs. Exposure of Titanium dioxide to the bacteria was decreased, leading to the formation of titanium nanoparticles. There was a change from light to dark brown in the color of the solution. The use of microorganisms and their metabolites as a tool for the synthesis of new functional nanomaterials has gained a lot of attention lately (16). A simple bio-biological method was used in the production of nanomaterials as an alternative to complex chemical synthesis processes. This process included the use of microorganisms i.e bacteria and fungi and the use of plant extracts (17). External polysaccharides and cell-free microbial cells may act as reducing agents in the biosynthesis of nanoparticles (18).

This study covered particle size was analyzed by Atomic Force Microscopy. Surface and 3D views of the nanoparticles were obtained by utilizing AFM, and it was found that the average size of particles was 18 nm while scanning electron microscope viewed as an oval shape with anatase form synthesized by *B. bifidum*. Analysis by TEM of a food-batch E171 titanium dioxide by the previous study. It was shown that 36% of the (number-based) molecules had a particle size smaller than 100 nanometers (19), also another study using electron microscopy (SEM) - analysis of 7 types of E171 titanium dioxide from the food type, this estimated that about 10 percent (number-based) of particles had a size of fewer than 100 nanometers (20).

Bifidobacteria strains are especially beneficial because of their improved ability to prevent pathogens of gastrointestinal through direct antimicrobial action and relieve colitis (21). A new method of synthesizing nanoparticles was proposed by (22). This method is characterized by a high capacity to produce nanoparticles at a low cost under ecofriendly circumstances and high-field production.

Antimicrobial activity of TiO2 NPS from bifidobacteria was examined and a well diffusion method using different concentrations (2000, 200, 100) mg was employed to determine the antibacterial activity of TiO2 NPS separated from *B. bifidum*. The diameter of inhibition zones around each well represented in (Table 1). Showed maximum zone of inhibition against *S. typhi, A. baumani, E. coli* and *P. aeruginosa* (28 mm) and *K. pneumonia* 26 mm at 200 mg and the least inhibition zone was observed against *P. aeruginosa* 18 mm, while equal inhibition zone showed by *A. baumani* and *K. pneumonia* 20 mm followed by *E. coli* and *S. typhi* 22 mm at 100 mg concentration. A study (23) reported that inhibition zone determination reveals that by concentration increasing of Tio2 against *E. coli*, the zones were also expanded (0, 0.2, 2.5, 3.0, 5.0) respectively with maximum inhibition zone (5mm) was noticed in 1.5% of nano-TiO2.

On the other hand, recorded that at one-hour exposure, nanoparticles gained ultra-inhibitory activity to *P. aeruginosa*, inhibition zone of isolate 1 (12 mm), isolate 2 (14 mm), isolate 3 (10 mm) and isolate 4 (8 mm) (24).

Several strains of Bifidobacterium, including commercial probiotics, have been examined to produce active antibacterial substances against Gram-negative bacteria. Bifidobacteria were found to engage in huge inhibition activity against Gram-negative bacteria, i.e intestinal Salmonella serovar Typhimurium SL1344 and *Escherichia coli* C1845. The inhibition mechanism examined and found to be dependent on lowering the pH in the medium and producing organic acids, especially acetic acid and lactic acid (25).

The produced photocatalyst TiO2 also exhibits photocatalytic activity against certain common pathogenic microorganisms i.e *E. coli, P. aeruginosa, Klebsiella pneumoniae* and *Staphylococcus aureus* are illuminated under visible light (26).

Metabolic activity of bacteria can be changed by metal oxide nanoparticles, and this capacity is a great advantage because it helps with the elimination of bacteria, which leads to the treatment of diseases (27). As found and reported by (28), bacterial decomposition of outer membranes by reactive oxygen species, initially hydroxyl radicals (OH), is responsible for the bactericidal effect of TiO_2, resulting in phospholipid peroxidation and finally death of cells. In addition, electrostatic interactions that disrupt bacteria functions cause nanoparticles to bind to the cell membrane or cell membrane proteins.

For each bacterium, the MIC was specified (Table 2). It was found that the MIC of TI-NPS against *S.typhi* and *E.coli* was 32mg/ml, whereas the MIC *for P. aeruginosa, A. baumanii, K. pneumonia* isolates were noticed at 16 mg/ml MIC. Variations in the tested genus and species possibly lead to differences in MIC of TiO_2 nanoparticles while (24) recorded TiO2 nanoparticles (0–500 mg / mL) demonstrated antibacterial activity against P. aeruginosa when pre-exposed with a one hour UV irradiation. Nanoparticles MIC for TiO2 were collected at 350 mg / mL.

As well as having important applications in the food

industry, Lactobacillus and bifidobacteria can have beneficial health effects as an adjuvant to decrease the intestinal microbiota imbalance caused by antibiotics or pathological conditions, particularly inflammatory intestinal disease (29). TiO_2 NPs allow bacterial fragmentation, degeneration, and compression of DNA, leading to a decrease in the physiological activity of genes. As a result, molecular docking predicted binding and affinity mode of nano-titanium dioxide and DNA, revealing that TiO_2 NPs targeted DNA is rich in GC (30).

The relationship between nanoparticles and radiation (31-40) as well as genes and cell biology (41-60) has been reported in many research. Also, hydrothermal synthesis of highly fluorescent and non-toxic carbon dots using Stevia rebaudiana Bertoni and nanoparticles synthesis by the green have been studied (61-62).

In our study, we reported that *B. bifidum* is an economical and eco-friendly method and a low cost and non-toxic biological approach that can be used to synthesize titanium dioxide nanoparticles. This trial presents a specific possibility to improve in the medical devices and materials. AFM and SCM were employed to characterize the formed titanium nanoparticles. Probiotic bacteria B. bifidum was used to obtain the particles with an average size of (81 nm) and were oval. These nanoparticles were found to have strong antimicrobial activity against a large number of pathogenic bacteria which induce acute diarrhea.

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Authors' contributions
KHIF was involved in the design and development of the research, carried out data analysis and part of practical FAAL participated in practical part and reviewing the initial and final drafts of the manuscript and SMIS performed the sampling and data collection. All authors read and approved the final manuscript.

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Availability of data and materials
On reasonable request, the datasets used for the current study are available. Please contact the respective author (second author), Fattma A.Ali for data requests. But I want to point out that part of the information was covered in another research more broadly regarding samples from patients and other tests that were conducted and do not include in this research and that will be submitted for publication in the future

Ethics approval and consent to participate
This study was accepted by the College of Health Sciences research ethics committee, Hawler Medical University, and administrative permission was obtained from Hawler Teaching Hospital. Stool samples were obtained from patients for participating in this study. Oral consent to participate in this study was obtained from the patient.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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