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Determination the antimicrobial activity of titanium dioxide nanoparticles synthesized from *Bacillus cereus* towards some multidrug resistant bacteria isolated from different sources of infection

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Abstract

The current study involved the manufacture of titanium dioxide nanoparticles (TiO_2 NPs) using *Bacillus cereus*. The microorganism was isolated, screened and characterized by morphological and biochemical analyses. UV-visible spectroscopy of the supernatant of cell culture showed absorbance peak of TiO_2 NP at ~ 353 nm. X-ray diffraction (XRD) techniques was used to characterize the particle size and morphology. Atomic Force Microscope (AFM) analysis shows the topography and roughness of the surface of TiO_2 NPs. The FTIR confirmed the existence of active groups as the stabilizing agent of the TiO_2 particles. Scanning Electron Microscope (SEM) observations revealed that synthesized TiO_2 NPs were spherical, oval in shape and irregular. The (TiO_2 NPs) were tested against antibacterial potential of some common human bacterial pathogens.

Keywords: Biosynthesis, TiO_2 NPs, *Bacillus cereus*, antibacterial activity

1- Introduction

At the present and future levels nanoparticles have received a great attention in the improvement of many sectors of the economy including solar energy products, transport, cosmetics, drugs and antimicrobial agents[1]. It is well known that many microorganisms, can produce inorganic materials by different ways either intra- or extracellularly[2]. Recent studies on the use of microorganisms in the synthesis of nanoparticles are a new and exciting area of research with considerable potential for development. A variety of techniques have been developed to synthesize metal nanoparticles including chemical synthesis[3] physical synthesis[4] and biological synthesis[5] . For biological synthesis of nanoparticles, plants and microbes have been exploited all over the globe. Microbes like bacteria, fungi and yeasts are mostly preferred for nanoparticles (NPs) synthesis because of their fast growth rate, easy cultivation and their ability to grow at ambient conditions of temperature, pH and pressure[6]. Microorganisms such as bacteria, can reduce heavy metal ions to produce nanoparticles. Researchers have demonstrated bacteria mediated interactive pathways responsible for metal ion reduction and their ability to precipitate metals on nanometer level[7].

The use of bacteria as a manufacturer of nanoparticles has a clear advantage because of the ease of manufacturing with minimal use of toxic chemicals, but there are some difficulties like laborious bacterial culturing processes and less control over their size, shape and distribution [8]. Fungi are recognized as eukaryotic organisms that reside in various ordinary lodgings and they typically form decomposer organisms. Yield of nanoparticles is high in fungi as compared to bacteria due to their toleration and metal bioaccumulation capability[9] and relatively larger biomass. *Bacillus cereus* is a Gram-positive, rod-shaped, aerobic, facultative anaerobic, motile, beta hemolytic bacterium commonly found in soil and food. Some strains are harmful to humans and cause foodborne illness, while other strains can be beneficial as probiotics for animals. Its virulence factors include cereolysin and phospholipase C[10]. These bacteria are metabolically chemoorganotrophs being dependent on organic compounds as sources of carbon and energy. In addition, genus *Bacillus* produces a variety of antibiotics, such as Zwittermicin A[11] , bacteriocin[12], cerein 7[13] and kanosamine[14]. Members of the *Bacillus* genus are generally found in soil and represent a wide range of physiological abilities, allowing the organism to grow in every environment and compete desirably with other organisms within the environment

due to its capability to form extremely resistant spores and produce metabolites that have antagonistic effects on other microorganisms[15] . The purpose of this current study was to evaluate the antimicrobial activity of synthesized TiO_2 NPs by *B. cereus*.

2- Materials and Methods

2-1 Isolation of *Bacillus cereus*

Samples of soils were randomly collected from Samarra University gardens. After the samples were collected and brought to the laboratory, 1 g of each sample of soil was added to a tube containing 9 mL of sterile distilled water. To select the spores, the serial dilutions were heated at 80ºC for 10 minutes to remove vegetative cells. 0.1 ml of each dilution was spread on nutrient agar plates (Himedia/ India). After an adequate duration of incubation growing colonies were inoculating on *Bacillus cereus* selective medium[16] and incubating for 24 h at 37ºC [17]. Resulted colonies were characterized according to [18]. Biomass preparation was done by inoculating one of *B. cereus* colony in 250 ml of MGYP broth medium (glucose 1%, malt extract 1%, yeast extract 0.3%, peptone 0.5%) and were incubated at 37.5°C in Shaking incubator (D.30938 GFL/ Germany) set at 100 rpm for 24 hours [19].

2-2 Synthesis of TiO_2 NPs

After incubation, the cell free filtrate was obtained and challenged with 0.025 M TiO_2 and stirred for one hour then the solution was heated at 60 ºC for 30 minutes. The white color deposit at the bottom of the flask gives a sign of TiO_2 NPs formation. The nanoparticles were separated by centrifugation and then dried. A negative control containing only TiO_2 and positive control containing a suspension of bacteria were maintained under similar conditions The biologically transformed particles were collected periodically and monitored for characterization[20].

2-3 Characterisation of Nanoparticles

2-3-1 UV–visible spectroscopy analysis:

Optical properties of TiO_2 NPs were measured by subjecting the sample to UV-Visible spectrophotometer (UV-1800 series-Shimadzu/ Japan), within the range 200 to 800 nm and absorbance was plotted on a graph[21]. (Samples were analyzed at Baghdad University, College of Education for Pure Sciences, Ibn Al- Haitham, Central Service Laboratory).

2-3-2 X Ray Diffraction analysis

Crystal structure, phase composition, phase purity and mean size of the nanoparticles are analyzed by X-Ray diffraction spectroscopy (XRD-6000- Shimadzu/ Japan). The X-Ray diffraction pattern of the synthesized nanoparticles were recorded between the range 10^0 to 90^0[22]. (Sample was analyzed at Baghdad University, College of Education for Pure Sciences, Ibn Al- Haitham, Central Service Laboratory.

2-3-3 Fourier transmission infrared spectroscopy (FTIR)

the sample was mixed with KBr and then pressed into thin pellet. Infrared spectra were measured using FTIR (Bruker/ Germany) at the wavelength in the range of 400-4000 cm^{-1}. This test was used to show the functional group of the TiO_2 nanoparticles[23]. (Sample was analyzed at University of Al-Nahrain, College of Science, Department of Chemistry)

2-3-4 Atomic force microscope (AFM)

Fifty microliters from the synthesized TiO_2 NPs solution was spotted on a slide then dried in an oven 40 ℃ for overnight. The surface topography of NPs was scanned and the size of NPs was calculated using atomic force microscope apparatus (Phywe measure nano/ England).(Sample was analyzed at University of Al-Nahrain, College of Science, Department of Chemistry)

2-3-5 Scanning electron microscopic analysis (SEM)

To characterize the morphology and the surface topology the synthesized nanaoparticles were coated with heavy metal gold and subjected to SEM imaging (Angstrom/ advanced/ USA). Thin films of the sample were prepared extra powder was removed and was subjected for SEM analysis. (Sample was analyzed at Baghdad University, College of Education for Pure Sciences, Ibn Al-Haitham, Central Service Laboratory).

3- Collection of pathogenic bacterial isolates

All pathogenic bacteria were obtained from the Salah El Din Hospitals and identified by experts using VITEK2 compact system. The pathogenic bacterial isolate were cultivated on selective media in the laboratory and some biochemical tests such as oxidase and

catalase were done. Also all the bacteria cells were stained by Grams stain for more confirmation. All the collected isolates were isolated from different site of infection.

3-1 Antimicrobial susceptibility patterns

Antibiotic susceptibility patterns of traditional and conventional antibiotics against bacterial isolates was interpreted as recommended by the Clinical and Laboratory Standards Institute (CLSI) by the disk-diffusion method [24]. Inhibition zone diameter (mm) of each antimicrobial disc was measured, and the isolates were classified as resistant and susceptible.

3-2 Antimicrobial activity of TiO_2 NPs

3-2-1 Well diffusion method

The antimicrobial activity of synthesized TiO_2 NPs was evaluated against the pathogenic isolates, 0.2 ml of fresh cultures of each organism was inoculated into 5 ml of sterile nutrient broth (Himedia/ India) and incubated for 3–5 h to standardize the culture to McFarland standards (10^6 CFC/ml). Three replicates of respective microorganisms were prepared by spreading 100 µl of revived culture on Mueller Hinton Agar media (Himedia/ India) with the help of spreader. Wells were made using gel puncture (6mm) according to[25], then 0.1ml of different dilutions of TiO_2 NPs (25, 50, 75 and 100%) were loaded in certain well. The plates were incubated at 37℃ for overnight. The zone of inhibition was measured (mm). This experiment was performed 3 times and then averages and standard deviations (SD) were calculated.

3-2-2 minimum inhibitory concentration (MIC)

Fresh overnight cultures of pathogenic bacterial isolates were adjusted to be about 1×10^8 cell per mL as mentioned above. One hundred twenty microliter bacterial culture from all isolates was placed in microtiter plate wells (96 microtiter plate). The 80 µL of appropriate dilution of TiO_2 NPs were added (25, 50, 75, 100 %). The control of this experiment was 200 µL bacterial culture only. The plates were incubated at 37℃ for 18 hours. The optical density (OD) at 600 nm was measured by using Microplate Spectrophotometer (Biotech µQuant™/ USA). This method was done according to [26]. Furthermore, five microliter was taken from each well and spotted on nutrient agar plates, then all plates were incubated at 37℃ for 18 hours. Growth percentage was calculated (see below formula) based on average and standard deviation (SD) of triplicate results.

Growth percentage % = OD of control – OD of treatment / OD of control × 100

4- Results and Discussions

4-1 Isolation and identification of *Bacillus cereus*

B. cereus group isolates showed typical colony morphology with a zone of precipitation, the isolates were Gram positive, rod shaped, endospore forming, catalase positive, lecithinase positive and hemolytic. all isolates were VP, Citrate, Gelatine positive.

4-2 Characterisation of Nanoparticles

The production of TiO_2 NPs by *Bacillus cereus* was characterized by UV-Visible spectroscopy. Figure (1) shows the absorption spectra at ~353 nm which are due to the surface Plasmon resonance /vibration in the reaction mixture. The absorption peak is an evidence for the formation of nanoparticle in the bacterial culture. This study coincided with many studies that indicated the absorption wave ranging from 200-800nm when manufacturing nanoparticles for titanium dioxide[27]. X-ray diffraction result confirmed the formation of nanoparticles. It is well known that each chemical has specific peaks appearing in the graph of the examined material. The strongest 3 peaks appear in the value of the diffraction angle (20-80) 2θ in (25.27, 37.76, 48.01) as shown in figure (2). The broader peaks indicate the smaller size of crystallite and vice versa due to random arrangement of crystallites[28]. The result of X-ray diffraction confirm the anatase crystal phase. Figure (3) showed the result of FTIR test which showed the absorption peaks of TiO_2 NPs synthesized by *B. cereus*. The occurrence of band at ~3276 cm^{-1} in the spectra can be assigned to the O−H stretching frequency arising from H bond. The impact of hydrogen bonding is to produce significant band broadening and to lower the mean absorption frequency. The lowering of the frequency tends to be a function of the degree and strength of the hydrogen bonding. The band at 2139 cm^{-1} was assigned to be C ≡ C terminal alkyne. Although acetylenic compounds are not very common, the spectrum associated with the C≡ C structure can be characteristic. It is instructive to note the impact on the carbon–carbon bond stretching as a function of increase in bond order for the series of single-, double-, and triple-bonded carbon. The appearance of characteristic bands 1635.28 cm^{-1} assignable to primary and secondary amine N– H stretching or organic nitrates. these structural differences are important, and they strongly influence the chemistry and the reactivity of the nitrogen and the N– H group (primary and secondary). Phenol or tertiary alcohol O–H bend in 1406.89 cm^{-1} or carboxylate (carboxylic acid) or may assigned to be organic sulfates. In compounds such as carboxylic acids, which exhibit extremely strong hydrogen bonding forming a stable dimeric structure, a highly characteristic, large shift to lower frequencies is observed. Also, the band at 1256 cm^{-1} is assigned to the aromatic primary amine (C– N) stretch. Also may represent organic phosphates (P–O) stretch. Peak at 626 cm^{-1} corresponds to C–H bending

vibration [29]. These observations accurately support the role of the presence of TiO_2 NPs synthesized from bacterial *B. cereus*. The results showed in figure (4-a,b) the downy surface of the biosynthesized TiO_2 NPs. AFM offers the capability of three dimensional visualization and both qualitative and quantitative information including Porosity, roughness, size, morphology, surface texture and fractal dimension were evaluated by analyzing the AFM images. A two and three-dimensional image was taken as well as the average size with 1548.16 nm^2 and average diameter 40.71 nm that were synthesized by *B. cereus* . For the two-dimensional images of the complex under study that the percentage of bright spots is a few, which represents the number of peaks located, which has the highest height and relative to the dark spots or different in the lighting, which have different ratios, which gives a heterogeneity of titanium oxide and microorganism. The SEM image clearly indicates the particles were agglomerated and they formed irregular Shape. Few particles with were spherical and oval in shape with 40.81 and 38.26 nm in size as shown in figure (5). Similar result of the TiO_2 NPs shape was reported by using *Lactobacillus* sp[30].

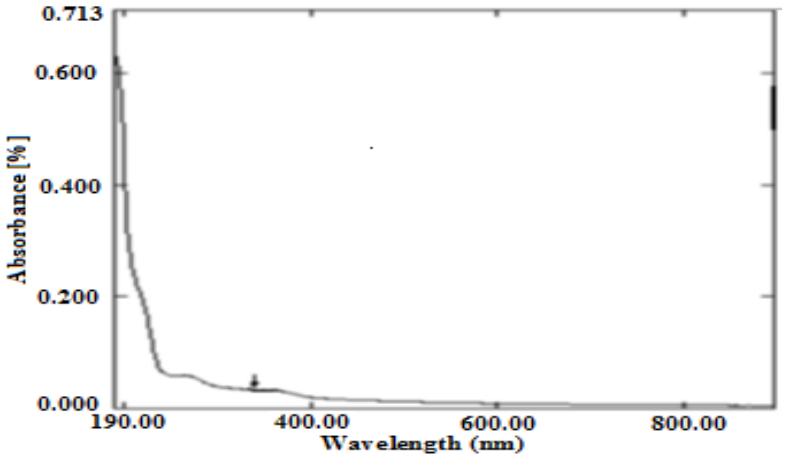


Figure (1) UV-Visible spectrum of titanium dioxide nanoparticles

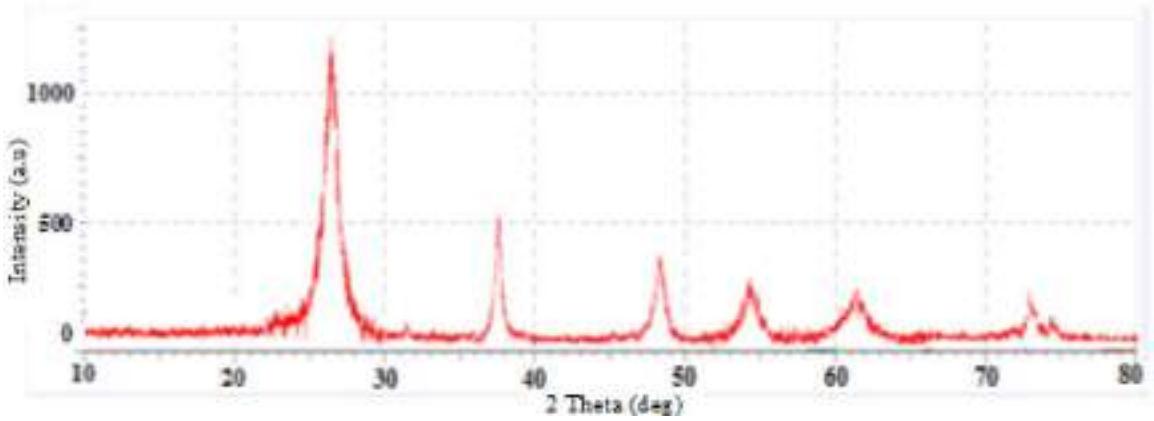


Figure (2) X-Ray diffraction pattern of TiO_2 NPs.

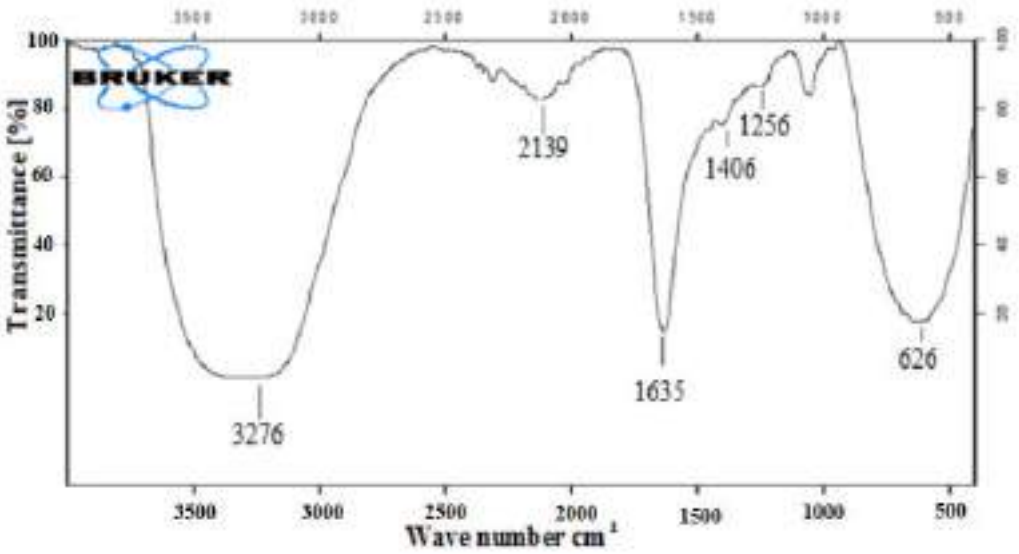
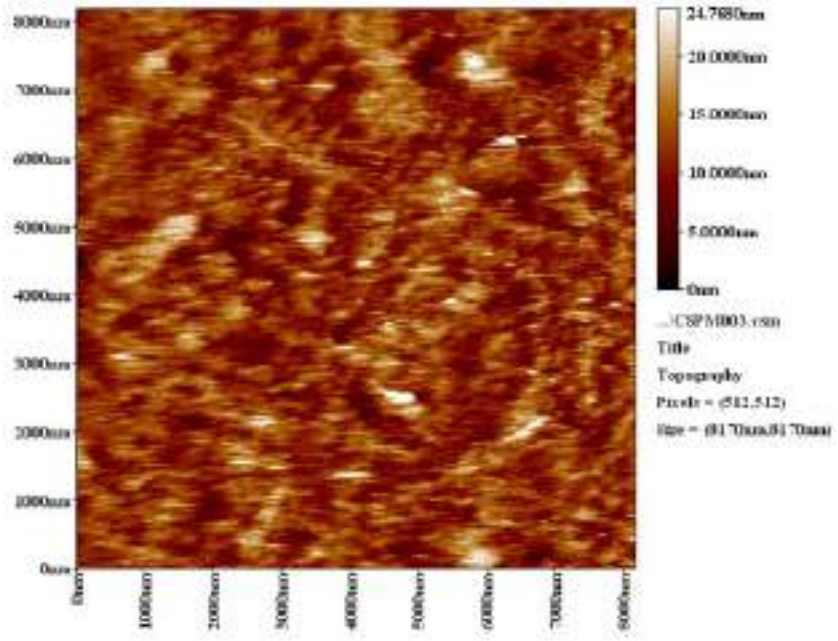
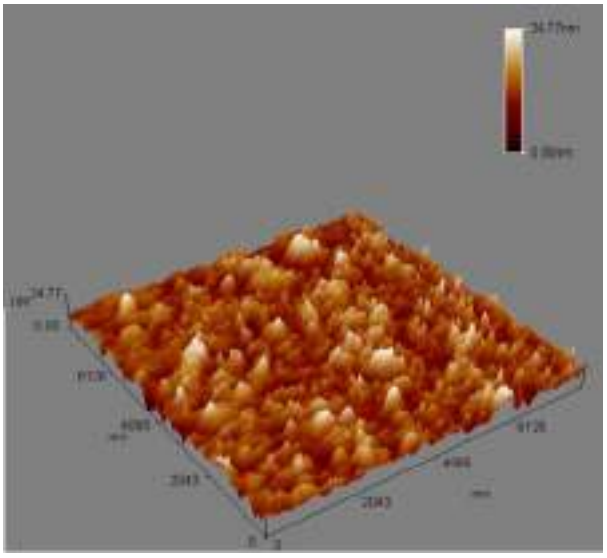


Figure (3) FTIR peaks of TiO_2 NPs.



a



b

Figure (4). AFM images of (a) topography of the surface of synthesized TiO_2 NPs by *B. cereus* at cross sectional view, and (b) top view of the synthesized TiO2 NPs particles.

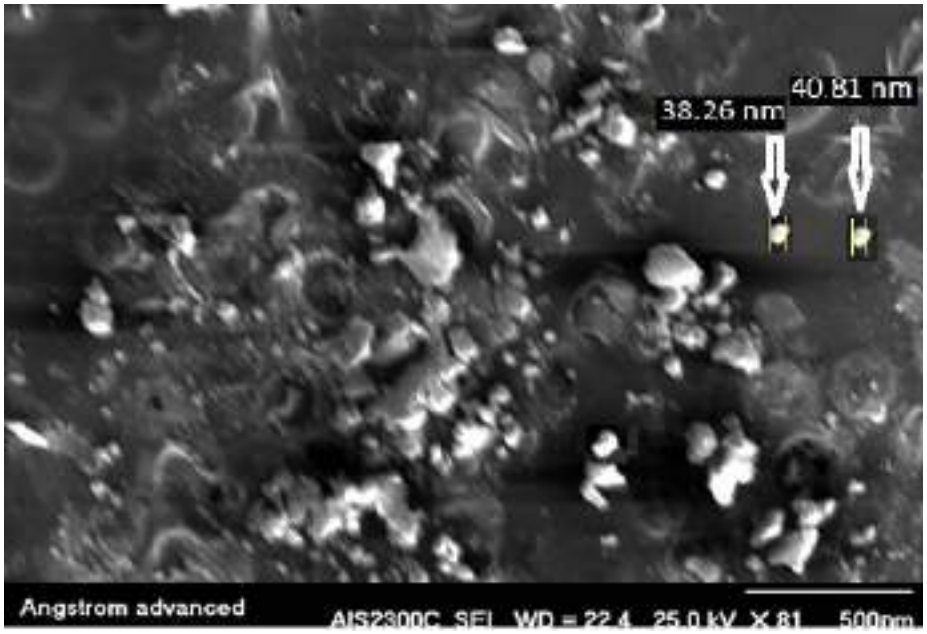


Figure (5). SEM images of synthesized TiO_2 NPs

4-3 Sensitivity of bacterial isolates to antibiotics

The sensitivity of bacterial isolates to antibiotics has been identified according to [24] . Table (1) shows that 100% of *E.coli* and *K. pneumoniae*, *P.aeruginosa, C. freundii* and *S. aureus* isolates were resistance to Augmentin, Azithromycin, Carbencillin, Ceftazidime and Clarithromycin while their resistance to Amikacin, Imipenem, Levofloxacin and Norfloxacin Tigecycline antibiotics was ranged from 0-80%.

Table (1) numbers and resistance percentage of bacterial isolates to antibiotics.

antibiotic bacteria	AK %	AMC %	ATH %	PY %	CAZ %	CLA %	IMP %	LEV %	NOR %	TGC %
E.coli *16*	13 81.25	16 100	16 100	16 100	16 100	16 100	2 12.5	7 43.75	8 50	10 62.5
K. pneumoniae 7	5 71.42	7 100	7 100	7 100	7 100	7 100	1 14.2	3 42.85	4 57.14	4 57.14
C. freundii 2	1 50	1 50	2 100	2 100	2 100	2 100	0 0.0	1 50	1 50	1 50
P. aeruginosa 2	2 100	2 100	2 100	2 100	2 100	2 100	0 0	0 0	1 50	1 50
S.aureus 3	2 66.6	1 33.3	2 66.6	3 100	3 100	3 100	0 0	2 66.6	1 33.3	2 66.6

Ak: Amikacin, AMC: Augmentin, ATH: Azithromycin, PY: Carbencillin, CAZ: Ceftazidime,
CLA: Clarithromycin, IMI: Imipenem, LEV: Levofloxacin, NOR: Norfloxacin, TGC: Tigecycline,

4-3-1 Agar diffusion and minimum inhibitory concentration (MIC)

Antibacterial test were performed using the well diffusion test [31] and MIC[32], each test was performed in triplicates. The well diffusion test showed a zone of inhibition in both Gram negative and positive. The higher value of inhibition zone was 8 mm against *S. aureus* whereas the minimum zone of inhibition was 4 mm against *P. aeruginosa* table (2). The MIC observed in the present study for synthesized TiO_2 NPs by *B. cereus* were (20) for *S. aureus* and *E. coli*, (80) for *P. aeruginosa*, (35) for *K. pneumoniae*, (50) for *C. freundii*. TiO_2 NPs can inhibit cellular enzymes and DNA by binding to the Electronic Donor Group such as carboxylic, amides, indole, hydroxyl, and sulfur groups. They also have the potential to make holes in bacterial cellular walls leading to increase permeability and cell death [33]. The results showed that TiO_2 NPs had an inhibitory effect against all negative and positive bacterial isolates. This was consistent with [34] who tested the inhibitor effect of TiO_2 NPs against the *E.coli* and *Enteroccocus faecalis* bacteria and observed no significant differences in their effectiveness against the Gram positive and Gram negative despite the structural differences of the wall of the cell. Consequently confirms that the structure of the cell wall is not the only factor in the interaction of bacteria to nanoparticles. The activity of TiO_2 NPs has been attributed to the decomposition of bacterial outer membranes by reactive oxygen species (ROS) which leads to phospholipid peroxidation and ultimately cell death [35]. The nanoparticles may react with the cellular base components namely sulphur and phosphorus. During this reaction, the sulphur and phosphorus content of genomic DNA possibly get affected by NPs which would definitely block DNA replication and thus kill the microbes [36]. The results were consistent with the findings of [37] who demonstrated that TiO_2 NPs had antibacterial activity under visible light conditions, as well as[38] indicated that TiO_2 NPs were the most biocompatible and antibacterial nanoparticles.

Table (2) Zone of inhibition (mm) and MIC mg ml^{-1} of *B.cereus* synthesized TiO_2 NPs against some pathogenic bacteria

Bacterial species	B.cereus synthesized TiO_2 Zone of inhibition (mm)	MIC mg/ml
E.coli	7.6± 0.4	20
K. pneumoniae	7± 0	35
C. freundii	7± 0.8	50
P. aeruginosa	4± 0.8	80
S. aureus	8± 0.4	20

5- Conclusion

The present investigation concludes that titanium dioxide nanoparticles can be successfully synthesized a simple and inexpensive nanocrystalline TiO_2 NPs powder using *B. cereus* at room temperature. The synthesized TiO_2 NPs were characterized by UV spectroscopy, XRD, FTIR, SEM and AFM studies, which concluded the formation of TiO_2 NPs. The antibacterial tests were performed and MIC values of the test bacterial species were tabulated. This technique will help in synthesis of other metal oxide by the said procedure in future.

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