Vol.2, No.3, Page 223- 231, 2024 https://doi.org/10.59628/jast.v2i3.792 ISSN 2958-9568 JAST

SUN PUBLISHER

مجلة جامعة صنعاء للعلوم التطبيقية والتكنولوجيا

Sana'a University Journal of Applied Sciences and Technology

https://journals.su.edu.ye/index.php/jast/

Biological Production of Titanium Dioxide Nanoparticles by a Combination Culture of *Lactobacillus* sp. and *Bacillus* sp. and Assessment of their Bioactivity

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Abstract

This research outlines the production of Titanium dioxide (TiO_2) nanoparticles (NPs) through the use of a combined culture of *Lactobacillus* sp. And *Bacillus* sp. The study evaluated the antibacterial performance of the TiO2 NPs against strains of Pseudomonas aeruginosa and Staphylococcus aureus that are resistant to multiple drugs. Characterization techniques such as UV-visible spectroscopy, X-ray diffraction (XRD), Fourier-transform infrared spectroscopy (FTIR), and transmission electron microscopy (TEM) were employed to analyze the TiO_2 NPs. The results indicate that the biosynthetic process leveraging a bacterial consortium is superior in both speed and efficiency to methods using a single type of bacteria. The XRD analysis substantiated the biological origin of the TiO_2 NPs, while FTIR spectroscopy suggested the existence of a protein layer enveloping the nanoparticles, potentially facilitating their synthesis and enhancing their stability. TEM imaging showed that the nanoparticles were spherical in shape with sizes ranging from 5.31 to 19.5 nm. Notably, the biologically synthesized TiO_2 NPs exhibited more potent antibacterial activity against *S. aureus* than against *P. aeruginosa*.

Article Info

Keywords:
Biosynthesis, Titanium Dioxide Nanoparticles, Mixture, *Lactobacillus*, *Bacillus*.

Article History:
Received: 12-December-2023,
Revised: 2-February-2024,
Accepted: 17-May-2024,
Available online: 30 June 2024.

1. INTRODUCTION

Nanotechnology is an accelerating advancing field in recent research, with broad applications in technology and science for the synthesis of new materials at the nanoscale [1]. Nanoparticles are created in different ways: biological, physical, and chemical, which alter the shape and size of the nanoparticles. Methods that are characterized by the absence of toxic chemicals, energy, high temperatures, and high pressure and have a lower cost for the synthesis of NPS are biological methods, which are distinguished from chemical methods [2]. Biological methods mainly use microorganisms or plant extracts for the synthesis of nanoparticles, containing proteins and polyphenols instead of chemical substances, as they reduce metal ions to nanoparticles [3]. Titanium dioxide is a non-toxic white dye used in many industries and environmental treatments. It is used in the manufacture of cosmetics, paints, ink, textiles, plastics, paper, and rubber, as well as in the photocatalytic destruction of pesticides using TiO_2 and water treatment [4, 5]. Metal oxide nanoparticles have been extensively studied over the years because of their potential applications in several fields [6]. Titanium dioxide nanoparticles (TiO_2 NPs) have unique electronic, optical, photocatalytic, and antimicrobial properties that can be used in new drug delivery methods as well as for water refinement, tincture-sensitized solar cells, photonics, and food conservation

[7, 8]. Metal ions converted into nanoparticles by plant extractors are faster than when using microorganisms [9]. However, the production of nanoparticles using plant extracts may necessitate the application of heat at a temperature of 85℃. This can result in significant expenses, particularly when manufacturing substantial quantities of nanoparticles [10]. Lack of access to raw materials and improper timing of plant harvesting can pose significant obstacles to the biosynthesis of nanoparticles by plant extracts [3]. Some plant raw materials require further processing before they can be used for the green synthesis of nanoparticles, which increases the complexity and cost of using plants in synthesis [3]. The utilization of a blend of diverse microorganism culture is becoming increasingly popular in various areas such as biomining, purification, fermentation, and bioelectricity production, as the employment of a solitary microorganism has proven to be ineffective [11]. Kang et al found that the effectiveness of a mixing of bacteria is more in reducing heavy metals is more efficacious than using single bacteria [12]. System of blended cultivation will be substantial to the progression of synthetic biology. The study of natural interaction between cells will shed light new procedures of remodulation even in organism that are complicate to cultivate [13]. The eventual goal of artificial biology is providing community benefit in the artificial, medicinal and environmental fields [12]. Numerous investigations have focused on the production of TiO_2 nanoparticles through bacterial biosynthesis utilizing a single bacterial genus. The use of a mixture of different bacterial species in the production of TiO_2 nanoparticles may enhance the ability to promote and accelerate synthesis processes. As a result, this current study delves into the production of TiO_2 nanoparticles using a combination of *Lactobacillus* sp. and *Bacillus* sp., and evaluating the antibacterial properties of TiO_2 nanoparticles versus multidrug-unsusceptible bacteria.

2. MATERIALS AND METHODS

2.1. Materials

Titanium tetrachloride, nutrient liquid medium and nutrient solid medium, Mueller–Hinton agar, MRS agar, simmon citrate medium, methyl red medium (MR), and Urease Indole Motility medium were got from Himedia Labs. sugars discs, hydrogen peroxide, Gram, and Ziehl–Nelseen smear. Sterilized normal solution (NS; 0.9 w/v% NaCl; pH 4.5–7.0). loops, swabs, cotton, and tubes. Deionized water (DW) was consumed whenever required.

2.2. Isolation and identification of *Lactobacillus* sp. and *Bacillus* sp.

Soil samples were gathered near a car reform shop in Dhamar Town, Yemen and transferred to the laboratory in sterile polythene sack. To create a soil solution, 10 grams of soil were diluted in 90 milliliters of sterile normal slain (0.9% w/v). Non-sporing bacteria were eliminated by subjecting the soil emulsion to 60℃ for one hour in a water bath. *Bacillus* sp. was cultured from every soil emulsion by streaking it onto nutrient agar medium and then incubated at 35℃ 24 hours. Additionally, an unprocessed milk sample from village Dhamar governorate was stocked in a sterile glass vial at 4℃ and transported to the lab. *Lactobacillus* sp. was insulated by outspreading the whey milk on De Man Rogos Sharpe medium with the pH adjusted to 5.4 using vinegar to facilitate the insulation of *Lactobacillus* sp. The bacteria were identified based on their morphological, biochemical, and physiological attributes following the proceedings outlined in Bergey's manual of methodical bacteriology [14].

2.3. Culturing the bacteria

Lactobacillus sp. and *Bacillus* sp. were separately introduced into two different sets of flasks: one set containing one hundred ml of nutrient liquid medium and the other set containing fifty ml of nutrient liquid medium. Subsequently, all the flasks were putted in an incubator at a temperature of 30 degrees Celsius and vibrate at 120 revolutions per minute for a duration of 24 hours.

2.4. Biosynthesized titanium dioxide nanoparticles

After the bacterial cultures reached the log phase, Titanium Tetrachloride was added to three separate flasks containing one hundred ml of *Lactobacillus* sp. outgrowth, one hundred ml of *Bacillus* sp. outgrowth, and one hundred ml of a blended culture of *Lactobacillus* sp. and *Bacillus* sp. The concentricity of Titanium Tetrachloride in every flask was 2 mM. The reaction was initiated by incubating the solutions at $30^{\circ}C$ with a vibrate at 140 rpm for 24 hours. This procedure was repeated twice. The formation of TiO_2 nanoparticles was indicated by color changes in the outgrowth solutions. Subsequently, the solutions were centrifuged at 10000 rpm for 20 minutes to detach the nanoparticles from other components. To ensure the purity of the NPs, they were laundered 3 times with DW. The nanoparticles were then dehydrated in a melting pot at $100^{\circ}C$ for 18 hours to obtain a fine powder suitable for further characterization.

2.5. Characterization

The TiO_2 NPs underwent analysis through a range of analytical methods A volume of five milliliters of the supernatant from the interaction solution was assessed using a UV-Vis spectrophotometer (Hitachi, Tokyo, Japan) at ambient temperature to ascertain the optical characteristics of the TiO_2 NPs. TiO_2 NPs were analyzed by X-ray

diffraction (XRD) using an XD–2 X-ray diffractometer (Beijing Purkinje General Instrument Co., Ltd., Beijing, China) together with CuKα radiation of $\lambda = 1.5418°$, scanning over a two-theta (2θ) range of 15°–75° at a rate of 0.02 min^{-1}. FTIR spectroscopy was employed to investigate the interactions amidst biomolecules and TiO_2 nanoparticles. The FTIR spectrum was recorded within the range of 400–4000 cm^{-1} with a resolution of 4 cm^{-1}. The morphology of the TiO_2 nanoparticles was examined using transmission electron microscopy (TEM) (JEM 100 CXII, Tokyo, Japan) at the Assiut EM Unit in Egypt. Diluted TiO_2 NPs were deposited upon carbon-plated copper TEM grids.

2.6. Antibacteria impact

The modified Kirby Bauer disk diffusion method [13] was exercised to investigate the antibacterial effects of TiO_2 NPs. The antibacterial assay was conducted using clinical specimens of multi-drug resistant *Staphylococcus aureus* (*S. aureus*) and *Pseudomonas aeruginosa* (*P. aeruginosa*), which were isolated from Al-Dubai laboratories for medical testing and scientific research in Dhamar city. The bacteria were activated by cultivating them on nutrient solid medium for 24 hours, and their cloudiness was adjusted to match that of barium sulphate (equal to McFarland 0.5) in sterile normal solution. Sterile swabs were used to inoculate Muller-Hinton agar plates with the bacterial turbidity. Subsequently, sterile filter sheet discs with a diameter of 6 millimeter, saturated with biosynthesized TiO_2 nanoparticles at different concentricities (10, 20, and 40 μg/mL), were aseptically putted on the Muller-Hinton agar plates. The diameter of the inhibition zone was measured in millimeters to calculate the extent of inhibition.

2.7. Hemolytic Assay

To appreciate the biosafety of TiO_2 NPs synthesized by bacteria, red blood cells were exposed to varying concentrations of the nanoparticles using a modified method based on a previous study [15]. Fresh blood (5 ml) from a healthy individual with B+ blood type was mixed with an anticoagulant in a tube. The erythrocytes were detached from the plasma via centrifugation at 6000 rpm for 8 minutes, followed by washing the RBCs three whets with normal saline. A 2% cell suspension of RBCs was intended in normal saline with a pH range of 4.5 to 7.0. TiO_2 nanoparticles were also dispersed in normal saline at concentrations ranging from 7.8 to 500 µg/ml. In each of nine tubes, 0.5 ml of the RBC suspension was added. Seven tubes received varying concentrations of TiO_2 nanoparticles, while tube No. 8 contained physiological solution as a negative control, and tube No. 9 had deionized water as a positive control. After incubating for 1 hour at 37℃ with kindly shaking, the tubes were centrifuged at 5000 rpm for 5 minutes. Hemolysis was assessed by measuring absorbance at 540 nm using a spectrometer [15]. The extent of hemolysis was calculated using the formula in this article [16].

3. RESULTS AND DISCUSSION

3.1. Characterization of isolated bacteria

The morphological analysis demonstrated that each the *Lactobacillus* sp. and *Bacillus* sp. cultures consisted of Gram-positive rod. Furthermore, the biochemical analysis, as indicated in Table 1, confirmed the presence of each *Lactobacillus* sp. and *Bacillus* sp.

Table 1. Characterization of morphological and biochemical features of *Lactobacillus* sp and *Bacillus* sp.

Test	*Lactobacillus* sp.	*Bacillus* sp.
Morphologic	Rod	Rod
Gram smear	+	+
Motile test	-	+
Catalase test	-	+
Spore forming	-	+
No aerobic culture	NT	-
Voges Proskauer (VP)	-	+
Citrate Use Test	NT	+
Egg yolk reaction (EYR)	NT	-
Carbohydrates Fermentation		
Arabinose	+	+
Lactose	+	NT
Mannose	+	NT
Mannitol	-	+
Sorbitol	+	NT
Fructose	-	NT
Galactose	-	NT
Xylose	NT	+

+ = positive; - = negative NT= Not tested

3.2. Synthesis of titanium dioxide nanoparticles

The mixed culture of *Lactobacillus* sp. and *Bacillus* sp. demonstrated the capability to biosynthesize TiO_2 NPs, as did the discrete cultures of *Lactobacillus* sp. and *Bacillus* sp. The forming of TiO_2 nanoparticles in the culture liquid was evidenced by the presence of a white precipitate of TiO_2 NPs [17], as depicted in Fig. 1. In the experiment, it was observed that the flask comprising a blend of *Lactobacillus* sp. and *Bacillus* sp. showed the formation of a white precipitate in less than 4 hours. On the other hand, in the flasks comprising *Lactobacillus* sp. and *Bacillus* sp. separately resulted in the appearance of a white precipitate after 18 hours. This highlights the advantage of using a blend of bacteria in biosynthesis,

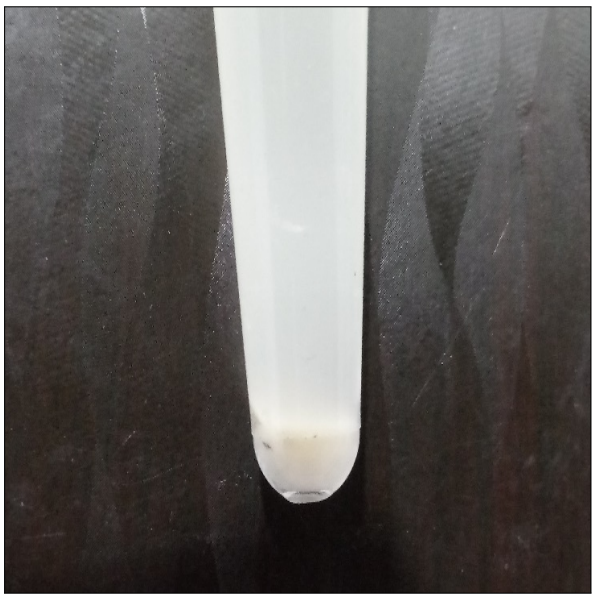

Figure 1. Shows the formation of a white precipitate indicates the bioformation TiO_2 NPs.

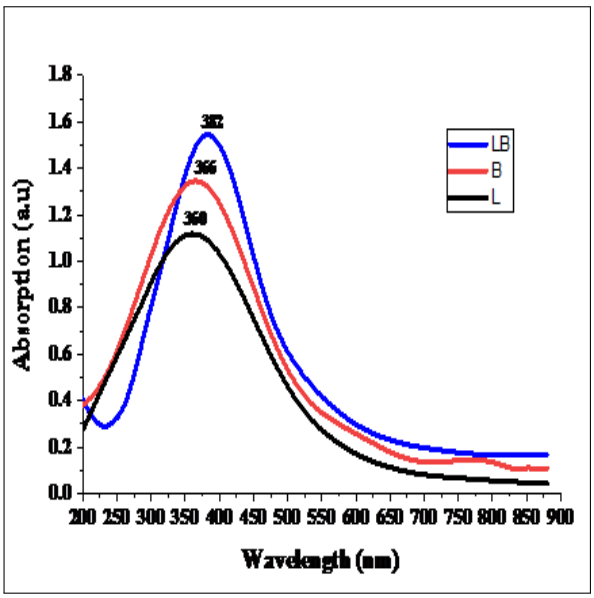

Figure 2. Displays the UV-Vis absorption spectra of TiO_2 NPs.

as it is swifter and more efficient outcomes compared to using a single bacterium. The increased efficiency of biosynthesis in mixed bacterial cultures can be attributed to several factors. Firstly, the metabolic byproducts of one bacterium can be harnessed via other bacteria, leading to a more efficient utilization of resources. Additionally, the interaction between different bacteria in the mixture enhances the overall effectiveness of biosynthesis [11, 18]. Furthermore, the mixed cultivation system offers several advantages over single cultivation systems, such as sturdiness, lower energy consumption, higher insensitivity to toxic substances, and higher productivity [19]. In comparison to single bacterial cultures, mixed bacterial cultures have been found to exhibit high reluctance and effectiveness in the bioremediation of heavy metals [20]. This further supports the notion that utilizing a mixture of bacteria in biosynthesis can yield superior results contrast to utilizing a solitary bacterium.

3.3. Characterization of TiO_2 nanoparticles

3.3.1. UV–visible analysis

The synthesis of TiO_2 NPs was analyzed through UV-visible spectrophotometer within the wavelength region spanning from 200 to 900 nm. The TiO_2nanoparticles produced by bacteria exhibited peaks at 360, 366, and 382 nm, attributed to their superficies plasmon reverberation absorption range [21], as illustrated in Fig. 2. Our findings align with prior research suggesting a typical TiO_2 NPs superficies plasmon reverberation absorption range falls within the wavelength region spanning from 300 – 400 nm [22, 23]. The symmetry of nanoparticles is reflected in the SPR peaks depicted in Fig. 2, where a higher degree of symmetry corresponds to a reduced number of SPR peaks [24].

3.3.2. X-ray diffraction

The crystalline properties and purity of TiO_2 NPs synthesized with the help of bacteria were assessed through X-ray diffraction (XRD) analysis. The diffraction peaks observed corresponded to the 2θ values of 27.4°, 35.82°, 39.9°, 43.5°, 52.82°, 56.52°, 63.6°, 68.6°, and 69.61°, which are associated with the (110), (101), (111), (210), (211), (220), (310), (301), and (112) planes of the standard cubic phase of TiO_2, as illustrated in Fig. 3. These diffraction peaks were in agreement with the standard data files (JCPDS card no: 21-1276) for all reflections [25]. The XRD analysis not only confirmed the pureness of the TiO_2 NPs but also indicated the obscurity of any undefined peaks within the range of 10° to 80°.

3.3.3. FTIR Analysis

The FTIR analysis was conducted on the dried TiO_2 nanoparticles in order to identify any protein surrounding the nanoparticles that could potentially contribute to the create and stabilization of the TiO_2 NPs. Figure 4 illustrates the FTIR spectra of the nanoparticles that were created by a combination of *Lactobacillus* sp. and *Bacillus* sp., as well as *Lactobacillus* sp., and *Bacillus* sp. individually. The recorded FTIR spectrum for the TiO_2 nanoparticles reveals that the peaks observed at 3433.05, 3432.67, and 3430.5 cm^{-1} may be attributed to the presence of the –NH or –OH group [26, 27]. Additionally, the peaks observed at 2934.54, 2924.56, 2920.3, 2851.13, 1384.44, 1383.68, and 1383.64 cm^{-1} indicate the presence of C–H functional groups [28, 29]. Furthermore, the band observed at 1735.62 cm^{-1} is likely due to the C=O group and aldehyde [29, 30]. The peaks at 1637.27, 1635.97 and 1627.63 cm-1 suggest the presence of NH bend in Primary amine, alkenyl C=C stretch,

Table 2. Antibacterial impact of TiO_2 NPs.

NPs	Bacteria	Inhibition area (mm) (mean ± SD of 3 replicates)		Control negative 40 µg/ml	
		10 µg/ml	20 µg/ml	40 µg/ml	
TiO_2 NPsfrom LB	*S. aureus*	10 ± 0.4	13 ± 0.1	16 ± 0.2	-
	P. aeruginosa	8 ± 0.56	11 ± 0.1	13 ± 0.22	-
TiO_2 NPs from L	*S. aureus*	9 ± 0.43	12 ± 0.24	14 ± 0.34	-
	P. aeruginosa	8 ± 0.76	10 ± 0.5	11 ± 0.3	-
TiO_2 NPs from B	*S. aureus*	8 ± 0.43	11 ± 0.12	13 ± 0.22	-
	P. aeruginosa	7 ± 0.23	9 ± 0.26	11 ± 0.27	-

LB = *Lactobacillus* and *Bacillus* L = *Lactobacillus* B = *Bacillus*

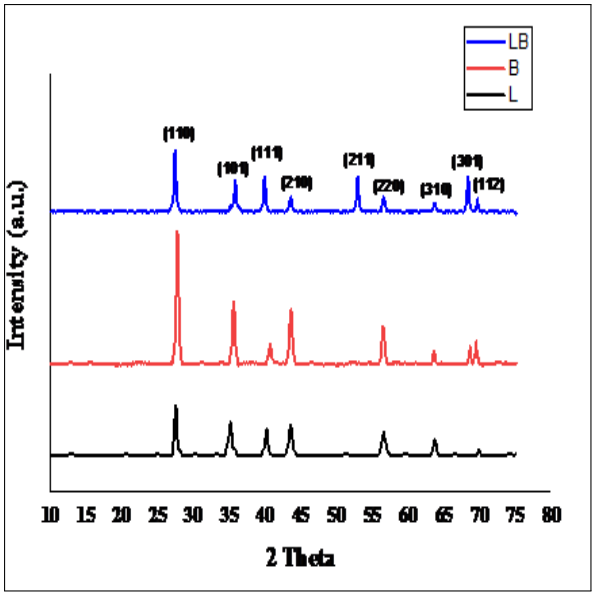

Figure 3. XRD spectra of TiO^2 NPs.

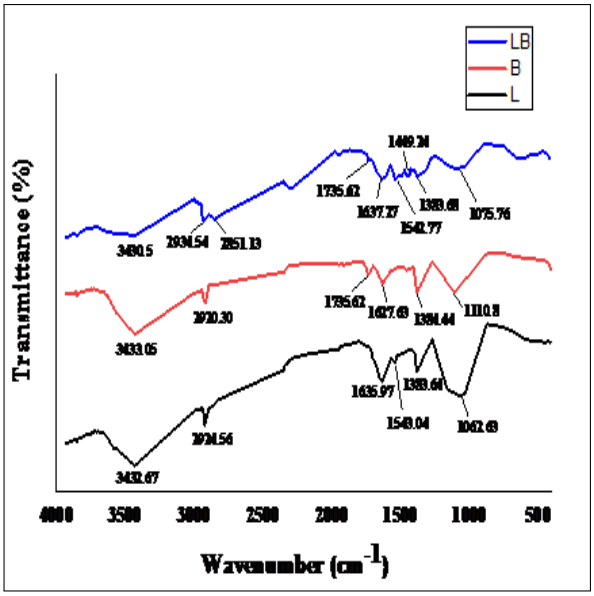

Figure 4. FT-IR spectrum of TiO_2 NPs.

and carbonyl in the amide [29, 31]. Moreover, the peaks at 1543.04 and 1542.77 cm^{-1} are allocated to C = C stretch in aromatic compounds [32]. The presence of a peak at 1449.24 cm^{-1} in the FTIR spectrum indicates the presence of CH_2 groups in proteins and lipids [33]. Additionally, the peak at 1110.8 cm^{-1} is attributed to the C–N expansion vibrations of aliphatic amines [17]. The bands observed at 1075.76 and 1062.63 cm^{-1} are indicative of the presence of CN stretch in Primary amine [2]. The FTIR results confirm the existence of a covering protein in the TiO_2 NPs derived from bacterial outgrowth. The create and stabilization of the TiO_2 nanoparticles can be attributed to the carboxyl group C=O, C–O bonds [34] and the free amine of bacterial protein.

3.3.4. TEM

The TiO_2 nanoparticles produced by bacteria are depicted in the TEM images shown in Fig. 5. The TEM micro diagram provides insights into the size distribution of these nanoparticles. Specifically, the size of the TiO_2 nanoparticles created through the growth of a mixture of *Lactobacillus* sp. and *Bacillus* sp. ranges from 5.31 to 9.01 nm. On the other hand, the nanoparticles synthesized solely by *Lactobacillus* sp. have a size range of 9.05 to 15.8 nm, while those synthesized by *Bacillus* sp. have a size range of 15.3 to 19.5 nm. The TEM image demonstrates that the TiO^2 nanoparticles exhibit a symmetrical spherical shape. Our findings align closely with the results obtained by Dhandapani et al., who observed that the nanoparticles synthesized using *Bacillus subtilis* (FJ460362) ranged in size from 15-20 nm [35]. The discrete distribution of TiO_2 nanoparticles, as observed in the TEM images, may be attributed to the presence of proteins, organic compounds, and secondary metabolites in the bacterial growth fluid, which could potentially cause clogging [36, 37].

3.4. Biological activity for TiO_2 NPs

The synthesized TiO_2 nanoparticles were assessed for their antibacterial properties versus multidrug-reluctant bacteria, specifically *Staphylococcus aureus* (*S. aureus*) and *Pseudomonas aeruginosa (P. aeruginosa*) obtained

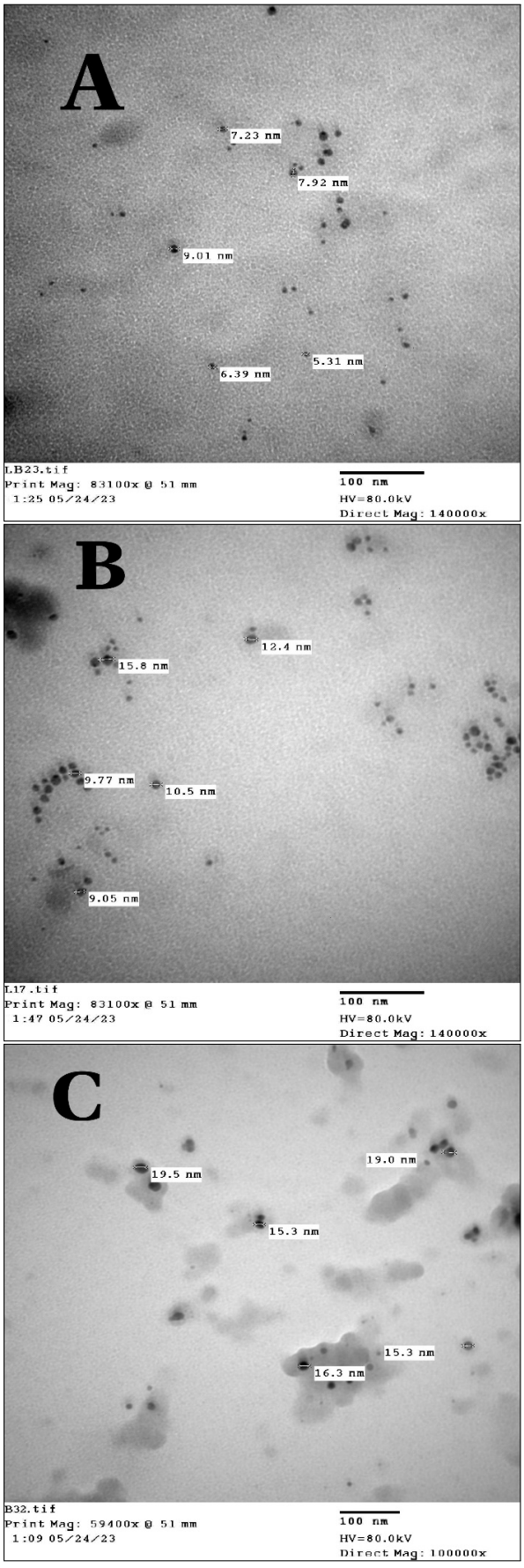

Figure 5. Displays TEM images of TiO_2 NPs synthesized using a combination of *Lactobacillus* sp. and *Bacillus* sp. growth in image (A), *Lactobacillus* sp. in image (B), and *Bacillus* sp. in image (C).

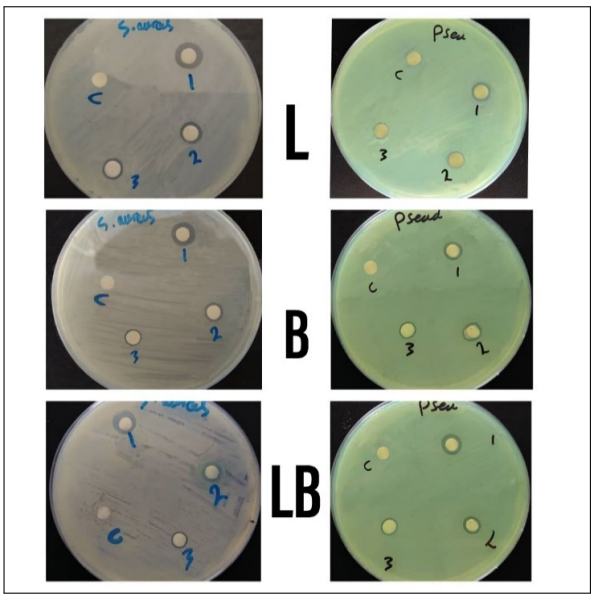

Figure 6. Some images for antibacterial impact of TiO_2 NPs versus *S. aureus* and *P. aeruginosa*. Discs: (1) 40 μg/ml, (2) 20 μg/ml, (3) 10 μg/ml and (c) control negative per discs.

from clinical specimens. The effectiveness of the antibacterial properties was assessed through the disc diffusion technique with varying concentricities of 10, 20, and 40 μg. The measurements of the inhibition area diameter (mm) around individual TiO_2 nanoparticle discs are detailed in Table 2. The antibacterial impact of TiO_2 NPs, produced through a blend of *Lactobacillus* sp. and *Bacillus* sp. as well as individually by each bacterium, exhibited effectiveness against *S. aureus* and *P. aeruginosa* as refer in Table 2 and Fig. 6. Our findings align closely with those of previous research studies [38]. The antibacterial feature of TiO_2 NPs are imputed to their crystalline structure, size, and shape. The detrimental effects on DNA, cell wall, and increased permeability of the biological membrane are induced by oxidative stress caused by reactive oxygen species (O_2^- , OH, and H_2O_2) [39, 40]. The antibacterial efficacy of TiO_2 nanoparticles created by bacteria was found to be higher versus Gram positive bacteria (*S. aureus*) compared to Gram negative bacteria (*P. aeruginosa*) in our study. This finding aligns with a prior investigation conducted by Al Masoudi et al. [25]. The reduced susceptibility of *Pseudomonas aeruginosa* to TiO_2 nanoparticles could be attributed to the existence of an external membrane in these Gram negative bacteria. The external membrane of Gram-negative bacterial cells is composed of a complex structure that includes phospholipids, lipoproteins, lipopolysaccharides, and a variety of proteins. The phospholipids found in the external membrane contain phosphorylated groups and serve as a region for absorption in these cells [41]. Metal deposition onto the external membrane is a strategy employed by Gram negative bacteria to mitigate the harmful effects of metals by restricting their diffusion through the cell membrane [42]. Through our research, it was discovered

that TiO_2 nanoparticles produced through a blend of *Lactobacillus* sp. and *Bacillus* sp. demonstrated increased antibacterial properties compared to TiO_2 nanoparticles produced by each bacterium individually as shown in Fig. 7. This enhanced efficacy can be attributed to the diminutive size of the TiO_2 NPs created by the bacterial blend. The potency of nanoparticles in eradicating bacteria and fungi is contingent upon their size, with smaller nanoparticles possessing a larger surface area, thus resulting in heightened effectiveness against bacteria and fungi when compared to larger nanoparticles [43].

3.5. Hemolytic of TiO_2 NPs

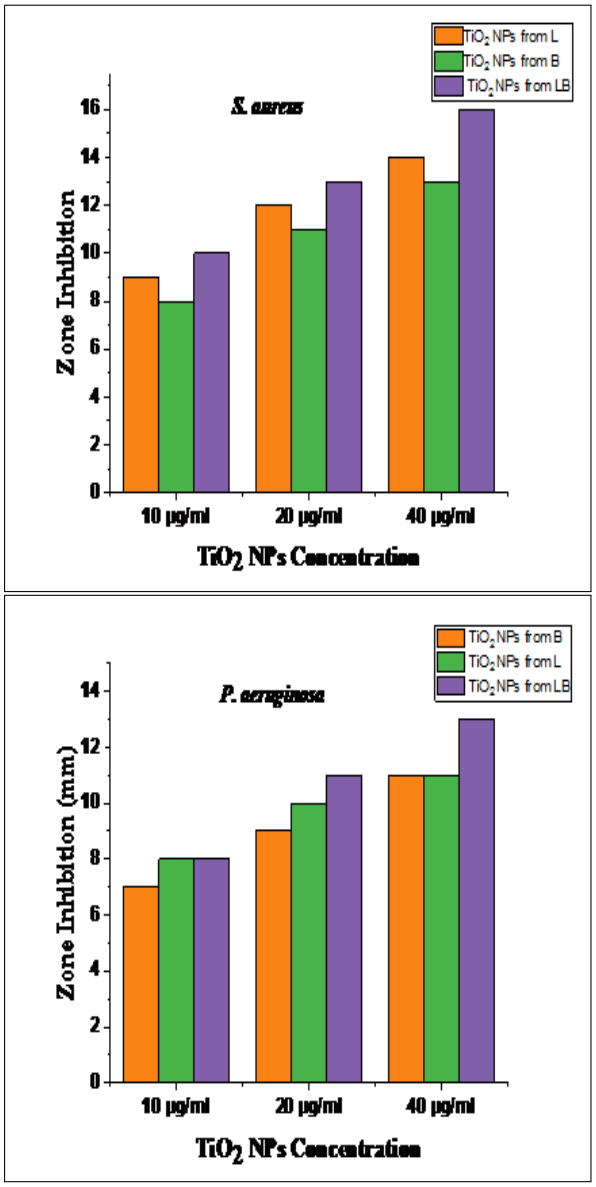

Figure 7. Illustration of the enhanced antibacterial effects of TiO_2 nanoparticles generated through the symbiotic cultivation of *Lactobacillus* sp. and *Bacillus* sp., compared to the nanoparticles synthesized by each bacterial species independently.

The hemolytic inspection was conducted using various concentricities of TiO_2 NPs (ranging from 7.8 to 500 μg/ml) on human erythrocytes, with normal saline serving as the negative control and deionized water as the positive control. Table 3 presents the mean data collected from the two experiments. In our investigation, the hemolysis of RBCs induced by TiO_2 NPs at concentricities of 500 µg/ml, 250 µg/ml, 125 µg/ml, 62.5 µg/ml, and 31.25 µg/ml was measured at 7.4%, 7.1%, 6.8%, 5.9%, and 5.7%, respectively. Conversely, minimal hemolysis was observed at concentrations of 15.62 µg/ml and 7.8 µg/ml, with rates of 4.7% and 1.8%, respectively. Substances causing hemolysis exceeding 5% were categorized as toxic, those inducing hemolysis between 2% and 5% were considered less toxic, and those leading to hemolysis below 2% were deemed non-toxic [44]. Our findings indicated that the TiO_2 nanoparticles exhibited no toxicity in comparison to the TiO_2 nanoparticles examined in the research conducted by Mahalakshmi S and Vijaya P [45], where they found that hemolysis at a concentration of 50 µg for TiO_2 NPs was 28.2%, 32.5 % at 100 µg, 39.2 % at 250 µg and 50.1 % at 500 µg [**?**], In their study, hemolysis rates of 28.2% at 50 µg, 32.5% at 100 µg, 39.2% at 250 µg, and 50.1% at 500 µg were reported, suggesting a high level of toxicity associated with the TiO_2 nanoparticles utilized, possibly attributed to their physical synthesis, as opposed to the bio-synthesized nanoparticles that demonstrated reduced toxicity [46].

Table 3. presents the hemolytic effects of titanium dioxide nanoparticles on red blood cells.

No	Transactional (µg/ml)	Hemolysis %
1.	500	7.4
2.	250	7.1
3.	125	6.8
4.	62.5	5.9
5.	31.25	5.7
6.	15.6	4.7
7.	7.8	1.3
8.	CN (NS)	0
9.	CP (DW)	100

CN= Control Negative; CP= Control Positive NS= Normal slain; DW= Distilled water

4. CONCLUSIONS

The current study has demonstrated that the blend of Lactobacillus sp. and Bacillus sp. for the synthesis of TiO2 nanoparticles resulted in a more efficient and rapid production process compared to using a single bacterial strain. Furthermore, these TiO2 nanoparticles exhibited improved antibacterial properties, especially against Staphylococcus aureus, in contrast to Pseudomonas aeruginosa. It is important to emphasize that the TiO2 nanoparticles produced in our investigation showed no

signs of toxicity, unlike previous research findings. Additionally, the TiO2 nanoparticles fabricated by bacteria in our experiment were considered to be safe and non-toxic.

ACKNOWLEDGEMENTS

The researchers express their gratitude to Dr. Salah Misfer, representing Al-Hikma University Dhamar, for providing access to its resources and laboratories, which were instrumental in conducting this study. Additionally, the authors extend their appreciation to Sabaa Bagah, Mayada Al-Asbahi, and Amani Al-Aghbari for their assistance in facilitating the research within the university's laboratory.

REFERENCES

[1] T. Santhoshkumar, A. Rahuman, C. Jayaseelan, *et al.*, "Green synthesis of titanium dioxide nanoparticles using psidium guajava extract and its antibacterial and antioxidant properties," Asian Pac. J. Trop. Med. **7**, 968–976 (2014).

[2] H. Al-Zahrani, A. El-Waseif, and D. El-Ghwas, "Biosynthesis and evaluation of TiO2 and ZnO nanoparticles from in vitro stimulation of lactobacillus johnsonii," J. Innov. Pharm. Biol. Sci. **5**, 16–20 (2018).

[3] S. Ying, Z. Guan, P. Ofoegbu, *et al.*, "Green synthesis of nanoparticles: Current developments and limitations," Environ. Technol. Innov. **26**, 1–20 (2022).

[4] R. Raliya, P. Biswas, and J. Tarafdar, "TiO2 nanoparticle biosynthesis and its physiological effect on mung bean (vigna radiata l.)," Biotechnol. Reports **5**, 22–26 (2015).

[5] G. Lusvardi, C. Barani, F. Giubertoni, and G. Paganelli, "Synthesis and characterization of TiO2 nanoparticles for the reduction of water pollutants," Materials **10**, 1–11 (2017).

[6] H. Alnahari, A. Al-Hammadi, A. Al-Sharabi, *et al.*, "Structural, morphological, optical, and antibacterial properties of CuO–Fe2O3–MgO–CuFe2O4 nanocomposite synthesized via auto-combustion route," J Mater Sci: Mater Electron **34**, 6821–6831 (2023).

[7] M. Peiris, T. Guansekera, P. Jayaweera, and S. Fernando, "TiO2 nanoparticles from baker's yeast: A potent antimicrobial," J. Microbiol. Biotechnol **28**, 1664–1670 (2018).

[8] S. Babitha and P. Korrapati, "Biosynthesis of titanium dioxide nanoparticles using a probiotic from coal fly ash effluent," Mater. Res. Bull. **48**, 4738–4742 (2013).

[9] H. Singh, M. Desimone, S. Pandya, *et al.*, "Revisiting the green synthesis of nanoparticles: Uncovering influences of plant extracts as reducing agents for enhanced synthesis efficiency and its biomedical applications," Int. J. Nanomed. **18**, 4727–4750 (2023).

[10] N. Pantidos and L. Horsfall, "Biological synthesis of metallic nanoparticles by bacteria, fungi and plants," J. Nanomed. Nanotechnol. **5**, 1–10 (2014).

[11] X. Li, C. Feng, M. Lei, *et al.*, "Bioremediation of organic/heavy metal contaminants by mixed cultures of microorganisms: A review," Open Chem. **20**, 462–467 (2022).

[12] C.-H. Kang, Y.-J. Kwon, and J.-S. So, "Bioremediation of heavy metals by using bacterial mixtures," Ecol. Eng. **89**, 64–69 (2016).

[13] L. Goers, P. Freemont, and K. M. Polizzi, "Co-culture systems and technologies: taking synthetic biology to the next level," J. The Royal Soc. Interface **11**, 20140065 (2014).

[14] P. Vos, G. Garrity, D. Jones, *et al.*, *Bergey's manual of systematic bacteriology: Volume 3: The Firmicutes*, vol. 3 (Springer Science & Business Media, 2011).

[15] A. Alnehia, A. Al-Sharabi, A. Al-Hammadi, *et al.*, "Structural, optical, and bioactivity properties of silver-doped zinc sulfide nanoparticles synthesized using plectranthus barbatus leaf extract," J. Chem. **2023**, 1–10 (2023).

[16] M. G. Al-Asbahi, B. A. Al-Ofiry, F. A. Saad, *et al.*, "Silver nanoparticles biosynthesis using mixture of lactobacillus sp. and bacillus sp. growth and their antibacterial activity," Sci. Reports **14**, 10224 (2024).

[17] S. Sunkar, V. Nachiyar, and K. Renugadevi, "Biogenesis of TiO2 nanoparticles using endophytic bacillus cereus," J. Nanoparticles Res. **16**, 1–11 (2014).

[18] F. Wu, B. J. Harper, and S. L. Harper, "Comparative dissolution, uptake, and toxicity of zinc oxide particles in individual aquatic species and mixed populations," Environ. toxicology chemistry **38**, 591–602 (2019).

[19] K. Jawed, S. Yazdani, and M. Koffas, "Advances in the development and application of microbial consortia for metabolic engineering," Metab. Eng. Commun. **9**, 1–10 (2019).

[20] K. Brenner, L. You, and F. Arnold, "Engineering microbial consortia: a new frontier in synthetic biology," Trends Biotechnol. **26**, 483–489 (2008).

[21] M. Altaf, M. Zeyad, M. Hashmi, *et al.*, "Effective inhibition and eradication of pathogenic biofilms by titanium dioxide nanoparticles synthesized using carum copticum extract," Royal Soc. Chem. **11**, 9248–19257 (2021).

[22] M. Taran, M. Rad, and M. Alavi, "Biosynthesis of TiO2 and ZnO nanoparticles by halomonas elongata ibrc-m 10214 in different conditions of medium," BioImpacts **8**, 81–89 (2018).

[23] K. Kotlhao, M. Madiseng, F. Mtunzi, *et al.*, "The synthesis of silver, zinc oxide and titanium dioxide nanoparticles and their antimicrobial activity," Adv. Mater. Proc. **2**, 479–484 (2017).

[24] M. Sowmya, A. Puttaswamy, H. Prasad, *et al.*, "Biogenic synthesis of alternanthera sessilis titanium dioxide nanoparticles (ASaTiO2NP's): A potential contender against perilous pathogens and catalytic degradation of organic dyes," Biointerface Res. Appl. chemistry **13**, 1–23 (2023).

[25] L. Masoudi, A. Alqurashi, A. Zaid, and H. Hamdi, "Characterization and biological studies of synthesized titanium dioxide nanoparticles from leaf extract of juniperus phoenicea (l.) growing in taif region, saudi arabia," Processes **11**, 1–15 (2023).

[26] T. Ahmed, Z. Wu, H. Jiang, *et al.*, "Bioinspired green synthesis of zinc oxide nanoparticles from a native bacillus cereus strain rnt6: Characterization and antibacterial activity against rice panicle blight pathogens burkholderia glumae and b. gladioli," Nanomaterials **11**, 1–15 (2021).

[27] D. El-Ghwas, "Short communication: Characterization and biological synthesis of zinc oxide nanoparticles by new strain of bacillus foraminis," Biodiversitas **23**, 548–553 (2022).

[28] A. Alali, A. Hosseini-Abari, A. Bahrami, and M. Mehr, "Biosynthesis of copper oxide and silver nanoparticles by bacillus spores and evaluation of the feasibility of their use in antimicrobial paints," Materials **16**, 1–13 (2023).

[29] A. Nandiyanto, R. Oktiani, and R. Ragadhita, "How to read and interpret ftir spectroscope of organic material," Indonesian J. Sci. Technol. **4**, 97–118 (2019).

[30] S. S. Suba, E. Vidhya, V. Punitha, and M. Nilavukkarasi, "Microbial mediated synthesis of ZnO nanoparticles derived fromn lactobacillus spp: Characterizations, antimicrobial and biocompatibility efficiencies," Sensors Int. **2**, 1–6 (2021).

[31] R. Khan and M. Fulekar, "Biosynthesis of titanium dioxide nanoparticles using bacillus amyloliquefaciens culture and enhancement of its photocatalytic activity for the degradation of a sulfonated textile dye reactive red 31," J. Colloid Interface Sci. **475**, 184–191 (2016).

[32] J. Santhoshkumar, S. Kumar, and S. Rajeshkumar, "Synthesis of zinc oxide nanoparticles using plant leaf extract against urinary tract infection pathogen," Resour. Technol. **3**, 459–465 (2017).

[33] A. Kirthi, A. Rahuman, G. Rajakumar, *et al.*, "Biosynthesis of titanium dioxide nanoparticles using bacterium bacillus subtilis," Mater. Lett. **65**, 2745–2747 (2011).

[34] A. Al-Odayni, A. Alnehia, A. Al-Sharabi, *et al.*, "Biofabrication of mg-doped ZnO nanostructures for hemolysis and antibacterial properties," Bioprocess Biosyst. Eng. **46**, 1817–1824 (2023).

[35] P. Dhandapani, S. Maruthamuthu, and G. Rajagopal, "Bio-mediated synthesis of TiO2 nanoparticles and its photocatalytic effect on aquatic biofilm," J. Photochem. Photobiol. B: Biol. **110**, 43–49 (2012).

[36] D. Anbumani, K. Dhandapani, J. Manoharan, *et al.*, "Green synthesis and antimicrobial efficacy of titanium dioxide nanoparticles using luffa acutangula leaf extract," J. King Saud Univ. – Sci. **34**, 101896 (2022).

[37] "Biosynthesis, characterizationm and optimization of TIO2 NANOPARTICLES BY NOVEL MARINE HALOPHILIC HALOMONAS SP. RAM2: APPLICATION OF NATURAL DYE-SENSITIZED SOLAR CELLS, author=Metwally, R.A. and Nady, J.E. and Ebrahim, S. and Sikaily, A.E. and El-Sersy, N.A. and Sabry, S.A. and Ghozlan, H.A., journal=Microbial Cell Factories, volume=22, year=2023, url=https://doi.org/10.1186/s12934-023-02093-3," .

[38] L. Abdulazeem, B. Al-Amiedi, H. A. Alrubaei, and Y. H. AL-Mawlah, "Titanium dioxide nanoparticles as antibacterial agents against some pathogenic bacteria," Drug Invent. Today **12**, 963–967 (2019).

[39] A. Khezerloua, M. Alizadeh-Sanib, M. Azizi-Lalabadib, and A. Ehsani, "Nanoparticles and their antimicrobial properties against pathogens including bacteria, fungi, parasites and viruses," Microb. Pathog. **123**, 505–526 (2018).

[40] Z. Zhua, H. Caia, and D.-W. Suna, "Titanium dioxide (tio2) photocatalysis technology for nonthermal inactivation of microorganisms in foods," Trends Food Sci. Technol. **75**, 23–35 (2018).

[41] A. Rapacka-Zdonczyk, A. Woźniak, B. Kruszewska, *et al.*, "Can gram-negative bacteria develop resistance to antimicrobial blue light treatment?" Int. J. Mol. Sci. **22**, 11579 (2021).

[42] P. Giovanella, L. Cabral, A. Costa, *et al.*, "Metal resistance mechanisms in gram-negative bacteria and their potential to remove hg in the presence of other metals," Ecotoxicol. Environ. Saf. **140**, 162–169 (2017).

[43] K. Lingaraju, R. Basavaraj, K. Jayanna, *et al.*, "Biocompatible fabrication of TiO2 nanoparticles: Antimicrobial, anticoagulant, antiplatelet, direct hemolytic and cytotoxicity properties," Inorg. Chem. Commun. **127**, 108505 (2021).

[44] A. Alnehia, A.-B. Al-Odayni, A. Al-Sharabi, *et al.*, "Pomegranate peel extract-mediated green synthesis of ZnO-NPS: Extract concentration-dependent structure, optical, and antibacterial activity," J. Chem. **2022**, 1–11 (2022).

[45] S. Mahalakshmi and P. Vijaya, "Evaluation of in-vitro biocompatibility and antimicrobial activities of titanium dioxide (TiO2) nanoparticles by hydrothermal method," Nano Biomed. Eng **13**, 36–43 (2021).

[46] M. Aravind, M. Amalanathan, and M. Mary, "Synthesis of TiO2 nanoparticles by chemical and green synthesis methods and their multifaceted properties," SN Appl. Sci. **3**, 1–10 (2021).