Optimization of Green Synthesis of TiO_2 Nanoparticles by *Bacillus velezensis* as Antibacterial Agent

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Abstract: *Streptococcus mutans* (*S. mutans*) being one of the most prevalent pathogenic bacteria, can form biofilms and cause oral infections. The subsequent need is a strong and effective antimicrobial material with minimal side effects to limit biofilm formation and biofilm proliferation from this bacterium. In this study we employed the Taguchi experimental design technique to study the synthesis of TiO_2 nanoparticles from *Bacillus velezensis* (*B. velezensis*) and evaluated their ability to inhibit the growth of *S. mutans* in vitro. Three main parameters influencing synthesis metatitanic acid concentration, glucose concentration, and incubation time were examined and analyzed at three different levels. Antibacterial effectiveness was maximally observed with the nanoparticles synthesized under the conditions of experiment 9 (1.5 mg/mL metatitanic acid, 3 mg/mL glucose and 48 hours incubation). It was observed that these nanoparticles were able to inhibit bacterial growth quite effectively and the bacterial viability was the lowest (0.31 Log_{10} CFU/mL) with these nanoparticles. With the outlined conditions of production, TiO_2 nanoparticles demonstrated considerable antimicrobial activity against *S. mutans*. The study indicates the promising ability of these nanoparticles in clinical therapies for the intervention and prophylaxis of biofilm-associated bacterial infections within the oral cavity.

Keywords: Streptococcus mutans, optimization, antibacterial, nanoparticles, biosynthesis

1. INTRODUCTION

Antimicrobial resistance (AMR) has been identified as a major global challenge in the field of human health (Safaei M, Taran, 2022). Even with advancements in oral health practices, dental caries continues to be prevalent. The emergence of multidrug-resistant (MDR) bacteria that can quickly form highly resistant biofilms is becoming a serious issue worldwide. More than 80% of microbial infections involve biofilm, and the increase in multidrug resistance makes their treatment harder. Thus, controlling them as part of public health and developing new antibacterial drugs and antibiotic alternatives to manage infections caused by pathogenic bacteria is important (Gurunathan *et al*., 2023).

Nanoparticles are conventionally defined as particles ranging from 1 to 100 nanometers. They have drawn a lot of press because their physical and chemical characteristics differ dramatically from bulk materials (Aslam *et al*., 2021; Safaei *et al*., 2025). The increase in the demand

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for these particles is primarily due to their high surface-to-volume ratio (SVR) and high surface energy, thus affording broad utility. Nanoparticles can be sorted into metallic or non-metallic categories based on elemental composition. TiO_2 nanoparticles have drawn special attention among metal oxide nanoparticles because of their superior biocompatibility, chemical inertness, photostability, and well-established safety profile in applications linked to dental care and biomedicine. TiO_2 is suitable option for applications that target oral infections like *Streptococcus mutans* because it has been approved for use in food additives, cosmetics, and dental goods. Metallic TiO_2 nanoparticles are considered superior for various applications because of their low production cost, high abundance, self-cleaning ability, strong oxidation power, and better chemical stability (Rathore *et al*., 2023).

The "bottom-up" and "top-down" methods correspond respectively to the assembly of atoms and the breaking down of bulk materials into nanoparticles. Recently, green synthesis of nanoparticles has received increasing attention. The desirable characteristics of nanoparticles synthesized via this method such as stability, catalytic activity, biocompatibility, high conductivity, and high SVR are among the reasons for this interest (Shimi *et al*., 2022; Serov *et al*., 2024). Bacteria are established as suitable agents for the generation of metallic nanoparticles due to their ability to reduce metal ions to metallic nanoparticles. Synthesis can occur via intracellular or extracellular methods; however, the extracellular method is the more used because of the advantages of genetic modification and lower costs (Jassal *et al*., 2022).

Microorganisms are regarded as potential "nanofactories" capable of producing nanoparticles in environmentally friendly, safe, and cost-effective ways without the need for harmful chemicals and with lower energy consumption. Microbial synthesis has a number of advantages over plant-mediated synthesis, such as easier downstream processing, shorter synthesis times, more control over nanoparticle size and shape, and reproducibility. Certain proteins, metabolites, and enzymes that function as stabilizing and reducing agents can be secreted by bacterial strains, allowing for the regulated production of nanoparticles in moderate environments. Biologically synthesized nanoparticles not only provide enhanced safety but also display outstanding biological activities. These nanoparticles play an important role in modern technologies with broad applications in environmental sciences, cell biology, agriculture, the food industry, and medicine (Revankar *et al*., 2022).

Due to their potential role in the synthesis, stability, and functionalization of nanoparticles, recent research has shown that plant growth-promoting bacteria, mostly *Bacillus* species, are extremely interesting candidates for microbial nanoparticle production. For instance, cyclic lipopeptides (LPs) with a hydrophilic peptide and hydrophobic lipid are produced by *B. velezensis*. The three primary groups of these LPs are fengycin, iturin, and surfactin. *B. velezensis* is an excellent biological candidate for the synthesis of green nanoparticles because these biomolecules are essential as bio-reducing and capping agents during nanoparticle formation. Nanotechnology has advanced so much recently that the biosynthesis of nanoparticles from lipopeptides is a budding field. The use of lipopeptides in the synthesis of nanoparticles from chitosan, iron oxide, silica, silver, zinc oxide, and aluminum silicate, all of which exhibit antimicrobial traits, indicates that *B. velezensis* might be a suitable biological candidate for the biosynthesis of nanoparticles. This provides an eco-friendly and low-risk approach to biosynthesizing nanoparticles for use in many different applications found in everyday life (George and Paari, 2023; Masmoudi *et al*., 2023).

The traditional ways of synthesizing TiO_2 nanoparticles are typically chemical or physical (Mohammadi *et al*., 2025). While they work, depends on toxic chemicals, have a large energy expenditure, and create dangerous waste (Sharifi *et al*., 2024). Moreover, they can be quite costly and usually require specific operational conditions that make them less feasible for large production scale. On the other hand, there has been increased interest in biosynthesis methods (using microorganisms, plants, or biological materials) due to their lower costs, being overall better for the environment, and process being simple. However, the literature on biosynthesis of TiO_2 nanoparticles has also mostly been limited to few microorganism strains, and at this time has overlooked to process the desirable nanoparticles size, shape, or to further enhance desirable antibacterial activity. These gaps show the need for more detailed systematic investigations while using and optimizing influential parameters from novel microorganisms (Torres-Liminana *et al*., 2022; Ansari and Daneshjou, 2024; Shiraz *et al*., 2024).

The purpose of this study is to improve the biosynthesis of TiO_2 nanoparticles using *B. velezensis* as an antibacterial agent against *S. mutans*. The study will investigate the impacts of various factors, including precursor concentrations and incubation time, on the size, morphology and antibacterial activity of the resulting nanoparticles. The overall objective is to achieve highly effective TiO_2 nanoparticles for antibacterial applications, while producing them using optimized synthesis conditions, which will allow for cost reduction in their production.

2. MATERIALS AND METHODS

2.1. Apply the Taguchi Method to Optimization Synthesis Condition

The Taguchi experimental design approach was used in this study and applied towards designing nine separate experiments for the synthesis of TiO_2 nanoparticles. The key variables included the metatitanic acid concentration, glucose concentration, to act as a carbon source and incubation time; each variable was tested at three distinct levels. The objective of this experimental design was to identify and optimize the synthesis parameters of TiO_2 nanoparticles with the best antimicrobial properties against *S. mutans* under in vitro conditions.

2.2. *Bacillus velezensis* Culture and Preparation

The *B. velezensis* strain PTCC 1927 which was used in this study was obtained from the Microbial Collection, Iranian Biological Resource Center (IBRC). This bacterial strain produces antibiotic compounds belonging to the aminoglycoside class. Nutrient agar medium was used for culturing and maintaining this microorganism at 30 °C and under aerobic conditions (Fazle Rabbee and Baek, 2020).

2.3. Optimization of the Biosynthesis of TiO_2 Nanoparticles

A nine-treatment experimental plan was established to optimize the production process of nanoparticles with maximum potential diameter, utilizing Qualitek-4 software, employing the Taguchi design method. Factors of metatitanic acid concentration (0.5, 1 and 1.5 mg/mL), glucose concentration (1, 2 and 3 mg/mL) and incubation times (24, 48 and 72 h) along with any interactions were assessed for main effects in a nine experiment design. The nine experimental design was performed in order to find optimum conditions for the biosynthesis of TiO_2 nanoparticles exhibiting potent inhibition against *S. mutans*, an oral pathogenic bacterium.

For production, bacterial cultures were prepared in glucose concentrations of 1 to 3 mg/mL. The cells were then placed into a broth with the different concentrations of glucose and incubated in the dark at 30 °C with shaking at 160 rpm for seven days. Harvesting the bacterial cells was done through centrifugation at 5000 rpm for 15 minutes to separate the supernatant and cell pellet. The supernatant was mixed with metatitanic acid solution was added to make up a 1:1 ratio to achieve 0.5, 1, and 1.5 mg/mL concentrations. The broths were then incubated in an orbital shaker at 30 °C, 200 rpm (Ghorbani *et al*., 2022).

2.4. Characterization

The prepared TiO_2 nanoparticles were characterized by Fourier-transform infrared spectroscopy (FTIR, Thermo Avatar, USA) which determined the structure and chemical composition. This analysis was done on the FTIR unit Thermo Avatar, where each sample was made into pellets and scanned using the IR light from 400–4000 cm^{-1} at room temperature.

The crystalline phase composition and crystallinity of the synthesized TiO_2 nanoparticles were determined using X-ray diffraction (XRD, Philips PW1730, Netherlands) operated at 40 kV and 30 mA. The samples were excited by X-ray with a defined wave length (Cu-Kα, λ = 1.54 Å). The collected data was collected in the 2θ. range of 20-80°. The obtained diffraction patterns were analyzed and the peaks were matched with information on standard crystal lattice spacing data to determine the crystallinity of the experimental samples.

The particle morphology and particles size distribution of the synthesized nanoparticles were evaluated through two forms of microscopy. The first process involved structural imaging using a scanning electron microscope (FESEM, TESCAN MIRA3, Czech Republic) at high resolution set to 30 kV. The second process involved a structural imaging evaluation through transmission electron microscopes (TEM, Philips EM208S, Netherlands) at an accelerating voltage of 100 kV.

To analyze the optical properties of the TiO_2 nanoparticles synthesized, UV-Vis spectroscopy (Thermo Scientific, USA) was employed. The absorbance spectra were determined using a Thermo spectrophotometer in the wavelength range of 200–800 nm. The nanoparticles were dispersed in distilled water at a concentration of 0.1 mg/mL, and the suspension was sonicated prior to measurement to ensure uniform dispersion. All spectral data obtained were analyzed in detail and this analysis enabled the study of the optical properties of the nanoparticles.

To identify and determine composition of the nanoparticles, energy-dispersive X-ray spectroscopy (EDX) was performed. After synthesizing, the nanoparticles were analyzed using an EDX system that was attached to a field-emission scanning microscope (Bruker, Germany). Elemental distribution maps were created to evaluate the uniformity of element distribution on the surface of synthesized TiO_2 nanoparticles.

2.5. Antibacterial Activity

For this research, to assess the antimicrobial properties of the synthesized TiO_2 nanoparticles against bacterial biofilms, *S. mutans* (ATCC 35668) was obtained from the microbial culture collection of the Iranian Industrial Microorganisms Resource Center. The bacteria were incubated for 24 hours on brain heart infusion agar plates in the standard laboratory conditions to obtain single colonies.

A bacterial suspension that corresponded to 0.5 McFarland standard concentration was then made. To facilitate biofilm formation, the bacterial suspension was transferred into dedicated wells of a 96-well plate and incubated at 37 °C for 72 hours. Every 24 hours, fresh medium (containing 2% sucrose and 1% mannose) was added into the wells to maintain optimal growing conditions.

At the end of the biofilm incubation period, samples were washed three times with PBS, to wash off planktonic cells.

Then, the synthesized TiO_2 nanoparticles that utilized the Taguchi experimental design procedure were added to each well (100 µg/mL) and incubated for 24 hours at 37 °C. Therefore, due to either solution or adherence, viable cells that remained in the biofilm, were collected from the walls of the wells, after incubation for 24 hours.

The cells that were biofilm-adherent were washed three times and resuspended in 1 mL of PBS. The resuspension was vortexed for two minutes to ensure mixing. The viable bacteria colony-forming units (Log_{10} CFU/mL) were determined by preparing the bacterial suspensions through a series of dilutions, culturing the dilutions on agar plates, and then incubating them for 24 hours at 37 °C. The colonies that formed were then counted, and the average from nine separate experiments, each corresponding to one Taguchi experimental condition and performed in triplicate, was calculated (Safaei and Moghadam, 2022; Safaei *et al*., 2022).

3. RESULTS AND DISCUSSION

3.1. FTIR Analysis

The FTIR spectrum of TiO_2 nanoparticles recorded between the 400–4000 cm^{-1} range is presented in Figure 1. For TiO_2 nanoparticles made using the bacterial method, the characteristic absorption bands usually emerged in the 400–800 cm^{-1} regions for the Ti–O vibrations, while the O–Ti–O bond was detected by the absorption peak at approximately 620 cm^{-1} (Bano, 2025). Broad absorption bands in the range of 3000–3700 cm^{-1} are usually related to hydroxyl groups, which may have resulted from water molecules or organic compound interactions during the synthesis method (Xiaoshang *et al*., 2021).

3.2. XRD Analysis

The diffraction peaks at 2θ of 25.49° (101), 38.14° (004), 48.34° (200), 54.24° (105), 55.34° (211), 62.7° (204), 69.39° (116), 70.74° (220), and 75.59° (215), agree with consistent standards illustrated in JCPDS card No. 89-4921 and show highly consistent with previously reported anatase TiO_2 reference patterns (Moradi Pour et al., 2022). The small additional peaks in the pattern may be a consequence of the proteinaceous layers around the particles (Hariharan *et al*., 2017; Jimoh *et al*., 2021).

3.3. FESEM Analysis

In Figure 3, an image of TiO_2 nanoparticles obtained simply by a field-emission scanning electron microscope (FESEM) at 70,000 magnifications with a scale bar of 500 nm is depicted, and demonstrates a clear image of the morphology and structural characteristics of the TiO_2 nanoparticles. As can be observed from the FESEM micrographs, the synthesized TiO_2 nanoparticles were uniformly distributed with minimal agglomeration, and were mostly spherical to oval in shape, having a

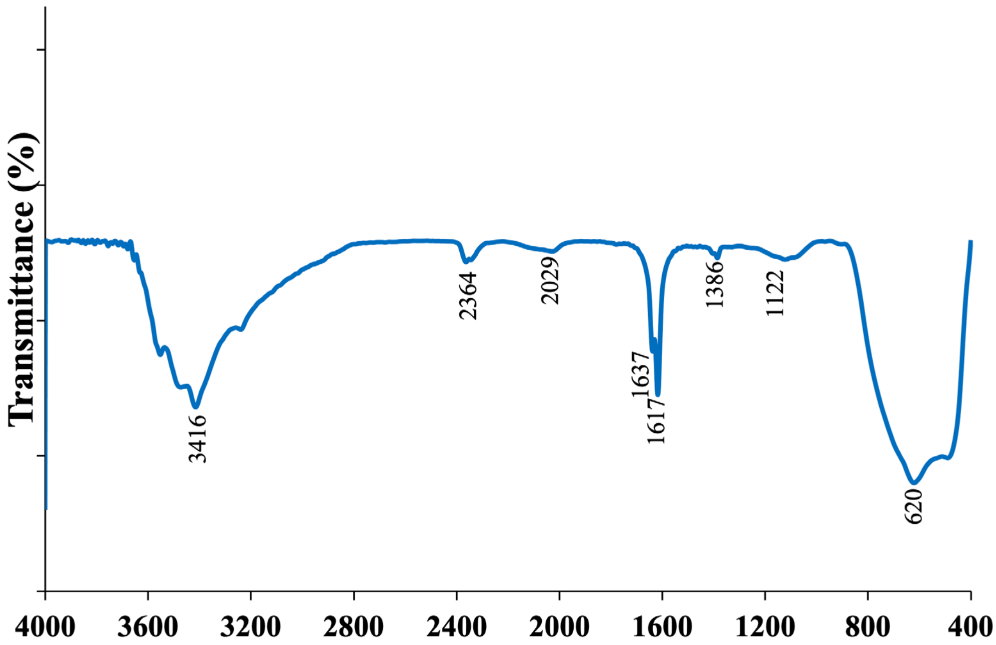

Figure 1. FTIR spectrum of TiO_2 nanoparticles biosynthesized by *B. velezensis*

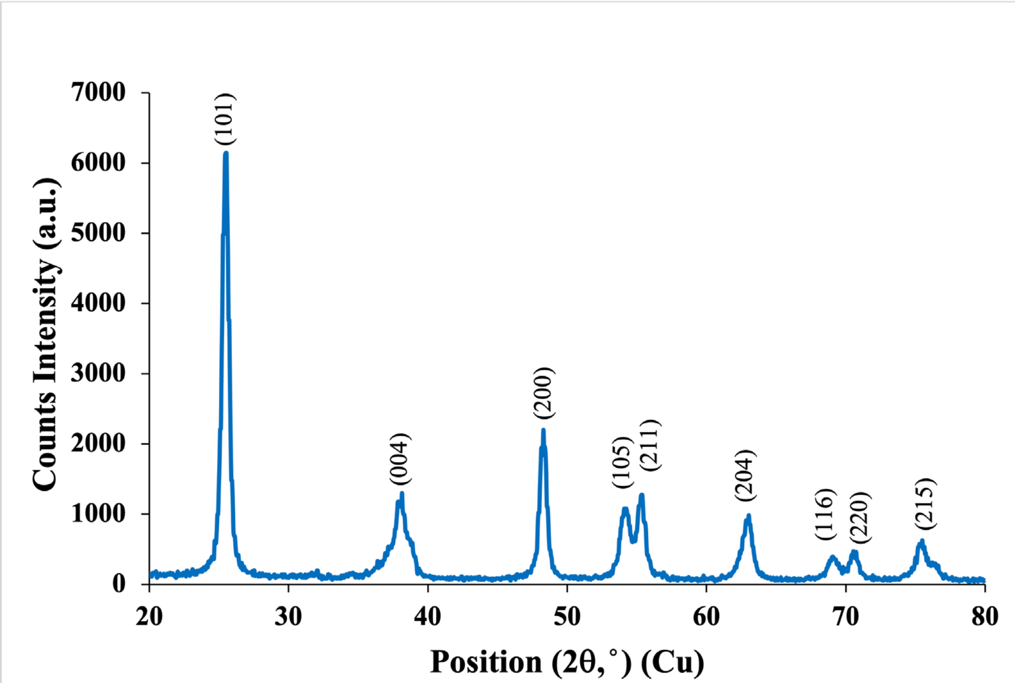

Figure 2. XRD pattern of TiO_2 nanoparticles biosynthesized by *B. velezensis*

relatively similar size. The well-defined particle boundaries, the lack of macroscopic contaminants, and an appropriate distribution in size all attest to the relative quality and structure of the nanoparticles. The image demonstrates that the synthesis of TiO_2 nanoparticles with relative physical and morphological characteristics, with appropriate parameter control, was achieved successfully.

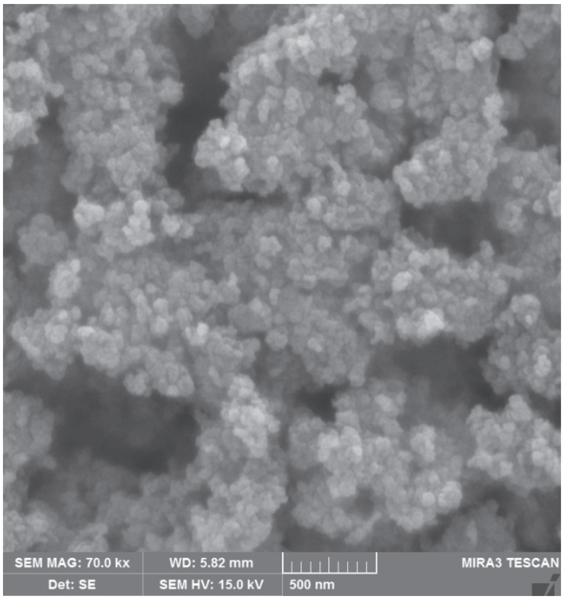

Figure 3. FESEM image of TiO_2 nanoparticles biosynthesized by *B. velezensis*

3.4. TEM Analysis

Figure 4 displays TiO_2 nanoparticles acquired via transmission electron microscopy (TEM). Most of the nanoparticles had a suitable size for biological applications and were smaller than 50 nm. This particle size range is consistent with previously reported biosynthesized TiO_2 nanoparticles, where particle sizes typically range from 10 to 50 nm, depending on the biological synthesis route and experimental conditions (Aslam *et al*., 2021; Ansari and Daneshjou, 2024). In the image, most of the nanoparticles were observed to be spherical or semi-spherical and uniformly that was observed is representative of a controlled synthesis process. Good dispersion of particles and an absence of sizeable agglomeration show good stability of the final product. These observations substantiate that the method of synthesis selected is capable of well physical characteristics of TiO_2 nanoparticles for the later applications intended.

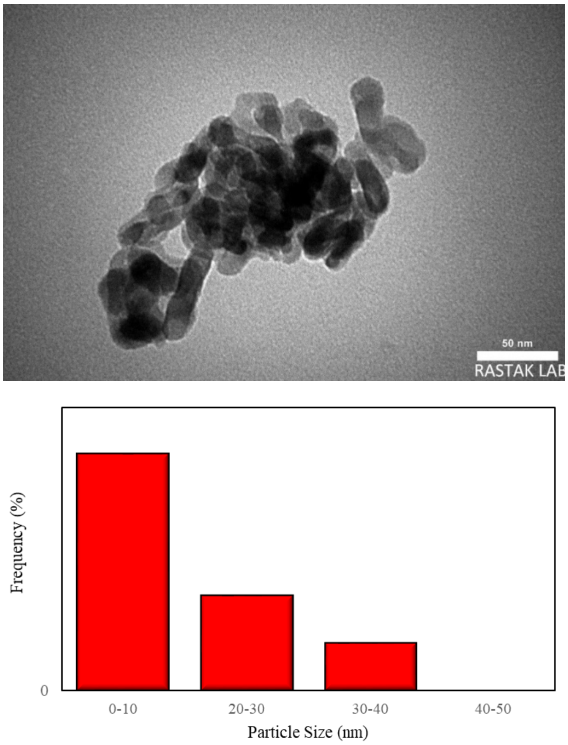

Figure 4. *TEM image of TiO_2 nanoparticles biosynthesized by B. velezensis*

3.5. UV-Vis Analysis

Absorption spectrum depicted in Figure 5 outlines the absorption spectrum of TiO_2 nanoparticles in the range of 200–800 nm. A distinct absorption peak is observed at approximately 330 nm, which is characteristic of anatase-phase TiO_2 nanoparticles. Previous studies have reported absorption peaks for these nanoparticles in the range of 300–350 nm (Enache *et al*., 2017; Saka *et al*., 2022). The UV-Vis spectrum indicating that essentially have a uniform distribution of particle sizes. Having obtained these results in tandem with the FESEM results that were previously discussed proved that we were able to achieve good control over the synthesis methodology parameters to produce nanoparticles with consistent optical and structural properties.

3.6. EDS Analysis

The energy dispersive x-ray spectroscopy (EDS) analysis in Figure 6, substantiated the presence of all constituent elements of TiO_2 nanoparticles. The quantitative results indicated an elemental composition by weight of 36.16% oxygen and 63.84% titanium, which aligns closely with the expected chemical formula (TiO_2). As a result, it is clear TiO_2 nanoparticles were formed, with the suitable chemical composition.

3.7. X-ray Mapping Analysis

Figure 7 shows the elemental surface distribution map of titanium dioxide nanoparticles, showing

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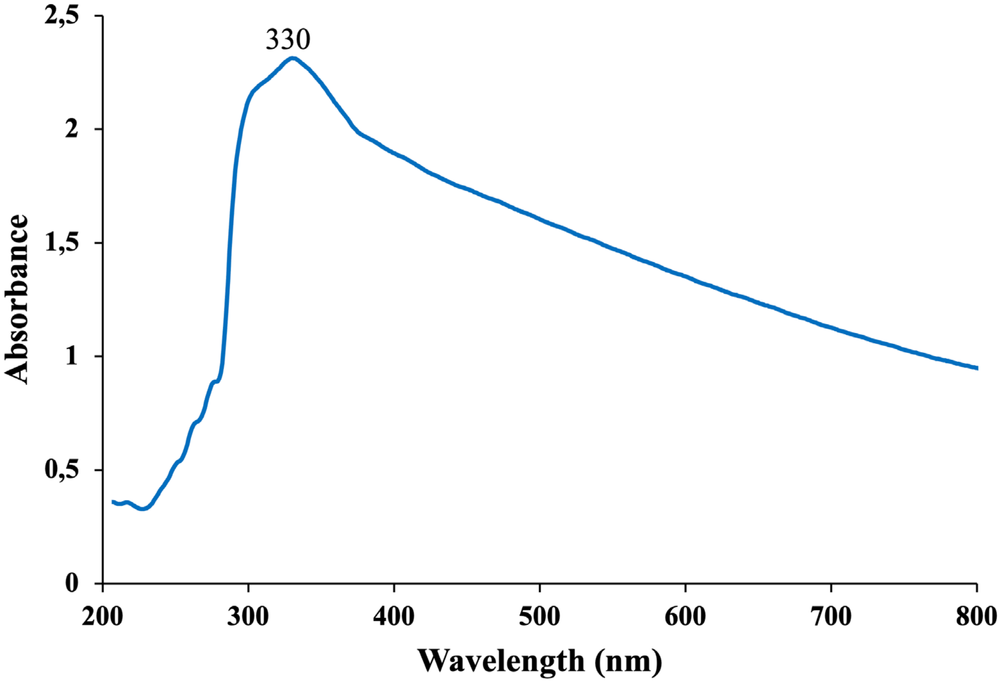

Figure 5. UV-Vis spectrum of TiO_2 nanoparticles biosynthesized by *B. velezensis*

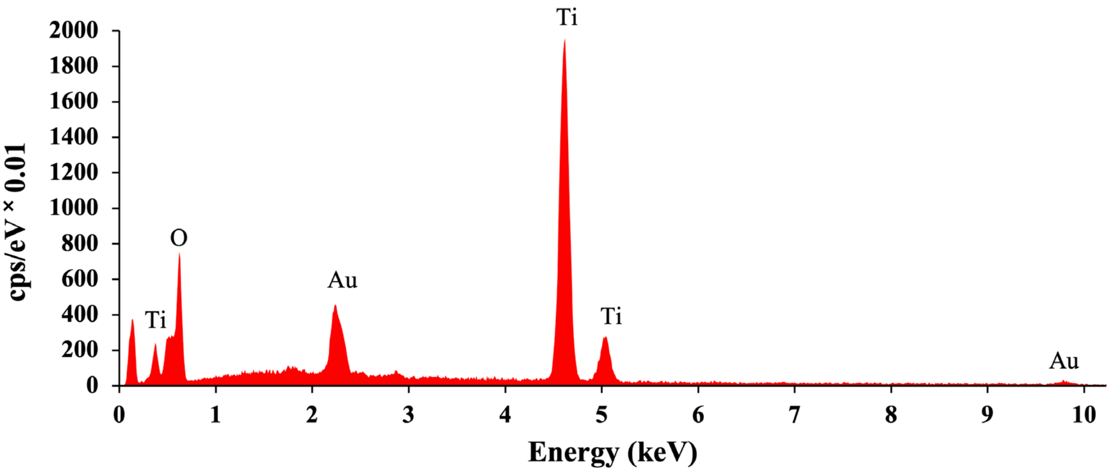

Figure 6. EDS spectrum of TiO_2 nanoparticles biosynthesized by *B. velezensis*

the evenly dispersed titanium and oxygen within the nanoparticle structure. This uniform pattern is confirmation that the synthesis process was successful, and there was uniform relative distribution of intermixed atoms in the generated nanoparticle structure. The results of this analysis show that titanium dioxide nanoparticles were successfully synthesized that had the correct composition and a uniform atomic distribution.

3.8. Antibacterial Activity

In order to find the optimal synthesis conditions for titanium dioxide nanoparticles at maximum antibacterial activity, nine experiments were undertaken using the Taguchi method. The effects of nanoparticles synthesized under different conditions to show bacterial survival were measured and shown in Table 1.

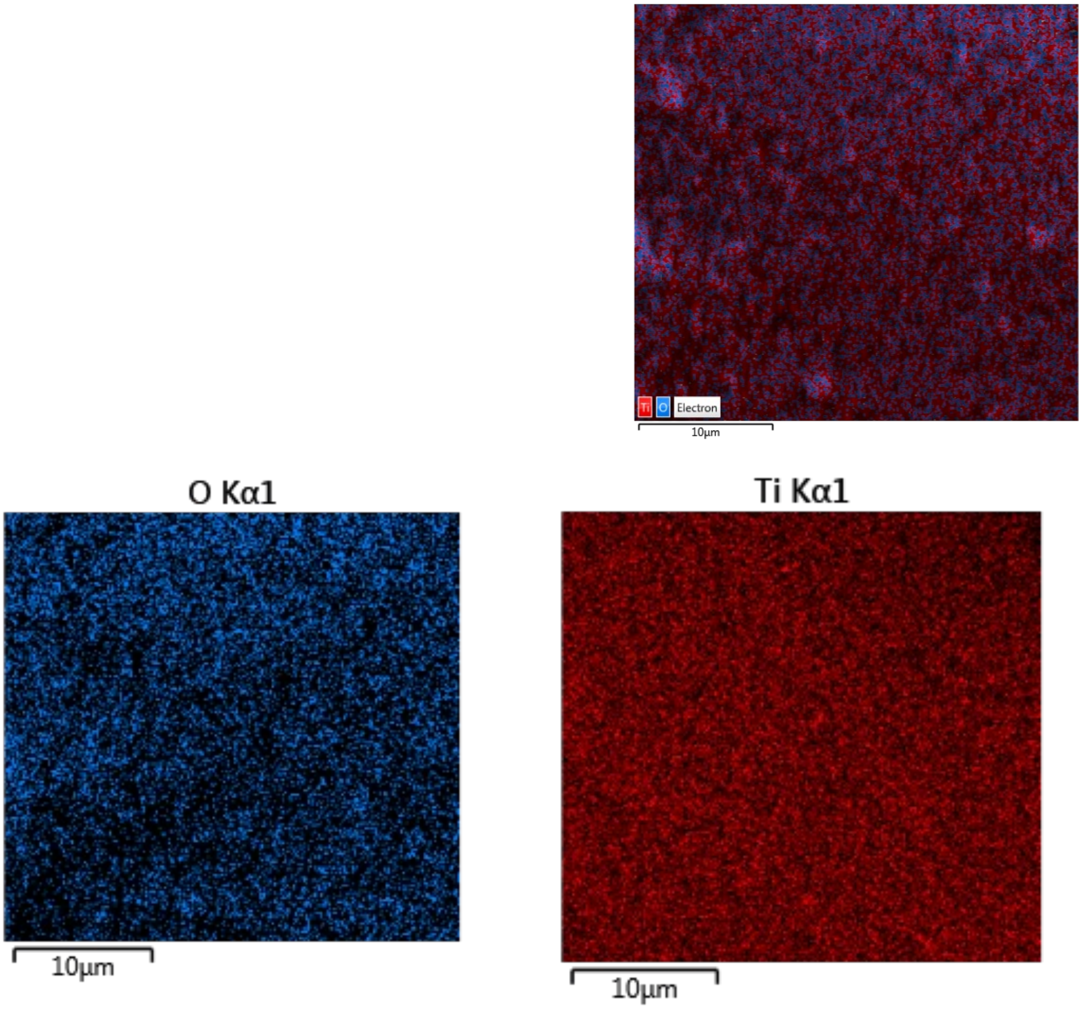

Figure 7. X-ray mapping of elemental distribution on titanium dioxide nanoparticle surfaces

Table 1. Taguchi design of experiments and bacterial growth inhibition rate of titanium dioxide biosynthesized nanoparticles

Experiment	Metatitanic Acid (mg/mL)			Glucose (mg/mL)			Incubation time (h)			Bacterial survival (Log_{10} CFU/mL)
	0.5	1	1.5	1	2	3	24	48	72	
1		0.5			1			24		2.72
2		0.5			2			48		1.67
3		0.5			3			72		0.78
4		1			1			48		0.91
5		1			2			72		0.45
6		1			3			24		0.76
7		1.5			1			72		0.52
8		1.5			2			24		0.61
9		1.5			3			48		0.31

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The experiment had the best results for antibacterial activity against S. mutans biofilms for nanoparticles synthesized using experiment No. 9; 1.5 mg/mL metatitanic acid, 3 mg/mL glucose, and 48 h incubation. The bacterial survival with those nanoparticles was measured as 0.31 Log_{10} CFU/mL, which was the lowest value among all the synthesized nanoparticles. The antibacterial activity observed in this study of biosynthesized TiO_2 nanoparticles is consistent with several previous studies (Younis et al., 2023; Ansari, F. S., & Daneshjou, 2024; Serov et al., 204). In this study, the use of the Taguchi optimization strategy highlights its improved efficacy in enhancing antibiofilm performance. Compared with non-optimized TiO_2 nanoparticles reported in the literature, the optimized nanoparticles developed in this study achieved significantly better survival values (Rahman et al., 2025), demonstrating the superiority of the optimization approach.

Pervious researches indicate that TiO_2 nanoparticles exert their antimicrobial properties through multiple mechanisms. The main mechanism is through the generation of reactive oxygen species (ROS) caused by light-activated nanoparticles. The ROS molecules, specifically superoxide radicals, attack the outer membrane proteins and lipids causing severe damage structurally leading to membrane damage and death of the microbial cells (Paul et al., 2023). The production of ROS increases membrane permeability, which causes intracellular materials to leak out leading to death of bacterial cells (Shandhiya et al., 2024). The membrane damage of bacterial cells leads to the leak of cytoplasm into the extracellular environment increasing cell death occurrence.

Another bactericidal mechanism of titanium dioxide nanoparticles is their direct interaction with microbial cell wall structure. Though the mechanism is not fully understood, their interaction alters the structures of surface components including proteins, carbohydrates, and membrane lipids. Nanoparticles can neutralize the surface charges on the bacterial membrane, significantly destabilizing the cellular membrane and promoting structural destabilization and loss of membrane integrity (Ziental et al., 2020). Studies indicate that titanium dioxide nanoparticles could maximize bactericidal effects through various methods. Reducing particle size dramatically increased antimicrobial potency. Additionally, the use of mineral doping, or introducing amorphous material to the nanoparticle structure can modify the properties of the nanoparticles and enhance the antibacterial performance of nanoparticles (Franco et al., 2022).

In Table 2, the effects of Metatitanic acid, glucose, and incubation time were displayed in relation to the survival of S. mutans. The results showed that, at Level 3, these levels had the most significant effect on the viability of the bacteria. Table 2. Main effects of different levels of Metatitanic Acid, Glucose, and Incubation time on the survival of S. mutans, respectively.

This study included interaction effects, as seen in Table 3. The interaction of Level 3 glucose and Level 2 incubation time combination had the largest combined effect at a bacterial survival of 59.54%. The combined effect of Level 3 metatitanic acid and Level 2 incubation time produced a significant effect of bacterial survival at 24.89% survival rate, but in comparison, the combination of Level 3 metatitanic acid and Level 3 glucose had the least effect at 12.24%.

Table 2. The main effects of different levels of Metatitanic acid, glucose and the incubation time on Bacterial survival of *S. mutans*

Factors	Level 1	Level 2	Level 3
Metatitanic Acid	1.72	0.71	0.48
Glucose	1.38	0.91	0.62
Incubation time	1.36	0.96	0.58

Table 3. The interaction effects of studied factors on bacterial survival of *S. mutans*

Interacting factor pairs	Column	Severity Index (%)	Optimum conditions
Glucose × Incubation time	2×3	59.54	[3,2]
Metatitanic Acid × Incubation time	1×3	24.89	[3,2]
Metatitanic Acid × Glucose	1×2	12.24	[3,3]

The analysis of variance (ANOVA) regarding the factors affecting the survival of *S. mutans* (Table 4) demonstrated that metatitanic acid had the greatest effect on bacterial viability (51.30%), followed by incubation time (14.55%) and glucose (14.23%) with all three factors having significant effects.

After considering all of the data and reviewing the factors and their interactions, the best conditions for synthesis of titanium dioxide nanoparticles with the most antibacterial activity were determined and are presented in Table 5. From these results metatitanic acid had the most effect on bacterial survival and glucose had the least effect. Incubation time had a more intermediate effect between the two. It was determined that Level 3 of metatitanic acid, glucose, and incubation time represented the best conditions for synthesis. The nanoparticles synthesized under these conditions inhibited bacterial activity down to 0.26 Log_{10} CFU/mL, which was affirming according to the results from Experiment No. 9.

4. CONCLUSION

In this research, a green synthesis of TiO_2 nanoparticles mediated by *B. velezensis* was developed and optimized for enhancing their antibiofilm activity against *S. mutans* using the Taguchi statistical design. The experiments in the Taguchi design proved to be an efficient approach in the optimization of the synthesis parameters, thereby leading to enhanced nanoparticles with great antibiofilm activity. Under the conditions of experiment 9 involving 1.5 mg/mL metatitanic acid, 3 mg/mL glucose, and 48 h incubation, the TiO_2 nanoparticles synthesized herein reduced bacterial survival to 0.31 Log_{10} CFU/mL. This study indicates that integrating green, bacteria-mediated synthesis with statistical optimization offers an efficient way to synthesize nontoxic antibacterial nanomaterials that may possess great potential for oral hygiene and dental biofilm control.

Table 4. Analysis of variance of factors affecting bacterial survival of *S. mutans*

Factors	DOF	Sum of Squares	Variance	F-Ratio (F)	Pure Sum	Percent (%)
Metatitanic Acid	2	2.63	1.32	11.30	2.40	51.30
Glucose	2	0.90	0.45	3.86	0.67	14.23
Incubation time	2	0.91	0.46	3.92	0.68	14.55
Other/Error	2	0.23	0.12	-	-	19.93

Table 5. Optimum conditions for the biosynthesis of titanium dioxide nanoparticles with the greatest antibacterial activity

Factors	Level	Contribution
Metatitanic Acid	3	0.49
Glucose	3	0.35
Incubation time	3	0.39
Total contribution from all factors		1.23
Current grand average of performance		0.97
Bacterial survival at optimum condition		-0.26

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