Biosynthesis of Chitosan Nanoparticles Via *Bacillus subtilis* for Effective Textile Effluent Wastewater Treatment and Antibacterial Activity

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Abstract **- This study investigates the use of Bacillus subtilis-synthesized chitosan nanoparticles (CNPs) for textile wastewater treatment and antimicrobial applications. Bacillus subtilis was isolated and used to produce CNPs, confirmed by UV-Vis spectroscopy with peaks at 325.6 nm. The textile effluent, with high pH and turbidity, showed significant improvements in physicochemical properties after CNP treatment, with the highest decolourization (63.0%) observed at 3 mL CNPs. The nanoparticles also exhibited dose-dependent antibacterial activity against Escherichia coli and Staphylococcus aureus, with larger inhibition zones for S. aureus (17 mm) compared to E. coli (15 mm). These results highlight the potential of Bacillus subtilis-synthesized CNPs as an effective and sustainable solution for both wastewater treatment and microbial control.**

Index Terms **- Bacillus subtilis, chitosan nanoparticles, textile wastewater, dye degradation, antimicrobial activity, nanoparticles synthesis.**

I. INTRODUCTION

The textile industry plays a crucial role in the global economy, contributing significantly to employment and GDP in many countries. However, it is also one of the most polluting industries, particularly in terms of water contamination. More than 3,600 individual textile dyes are manufactured by the industry today, and over 8,000 chemicals are used in various textile manufacturing processes, including dyeing and printing [1]. The industrial production of textiles is a long and complex process where natural or man-made fibers are converted into yarn and fabrics. The rise in hazardous dye wastewater generated by various industries poses a serious public health issue and a significant environmental concern, challenging conventional water treatment facilities [2]. The textile industry is a major generator of effluent wastewater due to its extensive water usage in wet processing operations. This wastewater contains chemicals such as acids, alkalis, dyes, hydrogen peroxide, starch, surfactants, dispersing agents, and metal soaps [3]. Water is the most vital resource for sustaining life, yet its availability and quality are under severe threat due to rapid industrialization, urbanization, and population growth. According to the United Nations, over 2 billion people live in water-stressed regions, and this number is expected to rise. The increasing discharge of textile effluents, laden with toxic dyes and chemicals, necessitates advanced treatment strategies [4]. Conventional methods, including physicochemical and biological approaches, often face efficiency, cost, and sustainability challenges [5].

In this context, nanotechnology, particularly chitosan nanoparticles, has gained attention for their adsorption capacity, biocompatibility, and antibacterial activity [6]. The textile industry alone is estimated to consume 93 billion cubic meters of water annually, accounting for 4% of global freshwater withdrawal. Textile wastewater contains complex mixtures of pollutants including dyes, suspended solids, heavy metals, and toxic chemicals [7]. Major pollutants include Synthetic Dyes Over 10,000 dyes are used in the textile industry. Approximately 20% of these are lost during dyeing, entering wastewater. These dyes are persistent due to complex aromatic structures, hindering light penetration in water bodies and affecting aquatic ecosystems [8]. Heavy Metals Dyes often contain metals such as Cr, Ni, Zn, Pb, Cu, and Cd, which are toxic and non-biodegradable, leading to bioaccumulation and long-term ecological damage [9]. Surfactants and Detergents: Used in scouring and washing, these contribute to high chemical oxygen demand (COD) and biological oxygen demand (BOD), reducing oxygen in water and harming aquatic life [8]. Recalcitrant Compounds: These

include dyes and chemicals resistant to biodegradation, persisting in water and soil [7].

Global Scenario The textile industry contributes to 20% of global wastewater, releasing 72 toxic chemicals into water bodies, 30 of which are non-biodegradable. The textile dye market is projected to reach $10.9 billion by 2027. Indian Scenario India is the second-largest textile exporter, generating 425 million litters of wastewater daily. Much of this is discharged untreated into rivers. Tamil Nadu Scenario Tamil Nadu, with hubs like Tirupur, generates 100 million litters of wastewater daily. The Noyyal River is heavily contaminated, impacting agriculture and public health. Chitosan, derived from chitin, is biodegradable, non-toxic, and biocompatible. It contains amino and hydroxyl groups that interact with pollutants through adsorption, chelation, and ion exchange [10]. Although bulk chitosan has limitations such as low surface area, converting it into nanoparticles enhances its properties.

Chitosan's cationic nature, especially at low pH, allows it to associate with negatively charged bacterial cell components, exhibiting antibacterial effects [11]. CNPs interfere with bacterial membrane and DNA functions, leading to cell death. Nanoparticles offer high surface area-to-volume ratios, improving adsorption efficiency [12]. Metal oxide NPs like hematite and MgO degrade pollutants effectively [13]. Biogenic NPs, especially chitosan nanoparticles (CNPs), are promising due to their environmental compatibility and multifunctionality. CNPs synthesized using Bacillus subtilis are efficient in pollutant removal and have antifungal properties [14]. CNPs can also achieve high dye removal efficiency, such as 94% Congo red adsorption using chitosan/fly ash composites [15]. Bacillus subtilis, a non-pathogenic, Gram-positive bacterium, is ideal for eco-friendly nanoparticles synthesis [16]. It secretes enzymes and proteins that reduce metal ions without harmful chemicals, producing stable nanoparticles with tailored surface properties [17].

The present study aimed to evaluate the effectiveness of Bacillus subtilis-synthesized chitosan nanoparticles (CNPs) in treating textile industry effluent and assessing their antibacterial activity. Due to the presence of harmful dyes, chemicals, and pathogenic microbes in textile wastewater, eco-friendly and efficient treatment methods are essential. This study investigates the potential of biogenic CNPs to enhance effluent quality by analysing changes in colour, pH, temperature, dissolved oxygen, and optical density, along with their antimicrobial efficacy, offering a sustainable solution for environmental remediation.

II. MATERIALS AND METHODS

A. Sample Collection

Soil samples were collected from textile effluent-contaminated areas in Erode, Tamil Nadu. The samples were aseptically collected using sterile polythene bags and transported to the laboratory. The samples were then used for isolation of *Bacillus subtilis*.

B. Isolation and Identification of Bacillus Subtilis

1 g of the soil sample was suspended in 10 mL of sterile distilled water and serially diluted up to 10□□. From the 10□³ and 10□□ dilutions, 0.1 mL aliquots were spread onto nutrient agar plates and incubated at 37°C for 24 hours. Colonies were examined for morphological features such as shape, size, colour, texture, and margin. Microscopic observations and Biochemical tests were performed as per Bergey's Manual of Determinative Bacteriology [18] for further characterization. Pure cultures were obtained by streaking selected colonies on nutrient agar plates, followed by incubation at 37°C for 24 hours. The pure isolates were then preserved at 4°C for further studies.

C. Biosynthesis of Chitosan Nanoparticle

Prepare a 1% glycolic acetic acid solution, 1 mL of glycolic acetic acid was added to 99 mL of distilled water. Chitosan stock solution was then prepared by dissolving 50 mg of chitosan powder in 50 mL of 1% glycolic acetic acid using a magnetic stirrer until a homogeneous solution was obtained. For bacterial culture preparation, Luria-Bertani (LB) broth was prepared using standard compositions. The medium was sterilized by autoclaving at 121°C for 15 minutes at 15 psi. A loopful of *Bacillus subtilis* was inoculated into 100 mL of sterile LB broth and incubated at 37°C for 24 hours under static condition. After incubation, the bacterial culture was centrifuged at 8000 rpm for 20 minutes at 4°C. The supernatant was discarded, and the pellet was retained for further use.

300 µL of chitosan stock solution was aseptically added to 100 mL of sterile LB broth. The previously

harvested bacterial cell pellet was then resuspended in the chitosan-supplemented LB broth and incubated at 30°C for 72 hours with continuous shaking at 180 rpm in a shaker incubator. The biosynthesis of nano-sized chitosan was confirmed by a visible colour change from pale yellow to dark brown, indicating successful nanoparticle formation.[19]

D. Extraction and Characterization of Chitosan Nanoparticles

After 72 hours of incubation, the culture medium was carefully transferred into 30 mL centrifuge tubes and subjected to centrifugation at 10,000 rpm for 10 minutes at 4°C to separate the biosynthesized chitosan nanoparticles from the reaction mixture. The resulting pellet, which contained the nanoparticles, was collected while the supernatant was discarded. To ensure the removal of residual impurities and unbound materials, the pellet was washed 3 to 4 times with sterile distilled water, and each wash was followed by centrifugation under the same conditions. After the final water wash, the purified pellet was resuspended in 30% to 70% ethanol and again centrifuged at 10,000 rpm for 10 minutes at 4°C. The ethanol step aided in further purification and stabilization of the nanoparticles. The final pellet was then resuspended in 10 mL of sterile distilled water, ensuring the nanoparticles were uniformly dispersed. This suspension was filtered through a 0.22 µm membrane filter to eliminate any remaining microbial contaminants or large particles. The clear filtrate containing purified chitosan nanoparticles (CNPs) was collected and stored at 4°C for further analysis.

The biosynthesized chitosan nanoparticles were characterized using UV-Visible spectroscopy to evaluate their optical properties. The absorbance spectrum of the nanoparticle-containing reaction mixture was recorded over a wavelength range of 300 to 700 nm. Prior to analysis, the sample was suitably diluted to ensure accurate and reliable spectral measurements. The UV-Visible spectrophotometer scan revealed characteristic absorption peaks indicative of nanoparticle formation, thereby confirming the successful synthesis of chitosan nanoparticles [20].

E. Collection and Analysis of Textile Effluent Water

The textile effluent water samples were collected from contaminated discharge points in textile processing units located in Erode. Sterile polyethylene containers were used for collection to avoid contamination. The containers were rinsed three times with the effluent before final sampling to ensure accuracy. Samples were taken directly from the outlet before mixing with any treatment or external water sources, to assess their original properties. After collection, the samples were immediately transported to the laboratory and stored at 4°C for further analysis.

Effluent samples were analysed for physico-chemical properties, including temperature measured with a thermometer, pH using pH paper, and colour observed visually. The dissolved oxygen (DO) concentration was also determined through titration with sodium thiosulfate. The results were recorded to assess the quality of the effluent water.

F. Textile Dye Degradation Using Chitosan Nanoparticles

The degradation of textile dye effluents was evaluated using chitosan nanoparticles (CNPs) synthesized by *Bacillus subtilis* in a batch experiment. Four 250 mL conical flasks, each containing 100 mL of textile dye effluent, were prepared with varying CNP concentrations: Flask 1 received 1 mL, Flask 2 received 2 mL, Flask 3 received 3 mL, and Flask 4 served as a control with no nanoparticles. The flasks were incubated at room temperature (28 ± 2°C) for 72 hours, with 5 mL samples withdrawn at 24, 48, and 72-hour intervals. After centrifugation at 10,000 rpm for 10 minutes to separate CNPs and suspended solids, the clear supernatant was analysed using a colorimeter at 550 nm. The absorbance measurements, with distilled water as the blank and the control as the reference, showed a decrease in absorbance over time, indicating the progressive breakdown of dye molecules facilitated by the chitosan nanoparticles [21].

G. Antibacterial Activity of Chitosan Nanoparticles

The antibacterial activity of chitosan nanoparticles against *Escherichia coli* and *Staphylococcus aureus* was assessed using the well diffusion method under aerobic conditions. Overnight bacterial cultures were grown in broth, and Muller-Hinton Agar (MHA) plates were prepared and inoculated with 100 µL of each bacterial suspension using the spread plate method for uniform distribution. After the plates dried, uniform wells (7.0 mm) were made using a sterile cork borer. Four wells were created: one for the control (sterile distilled water) and the other three for 50 µL, 100 µL, and 150 µL of chitosan

nanoparticle solution to assess the concentration-dependent effect. The plates were incubated at 37°C for 24 hours, and the antibacterial activity was determined by measuring the zone of inhibition around the wells, indicating the effectiveness of chitosan nanoparticles against the bacterial strains [22].

III. RESULTS

A. Morphological Identification

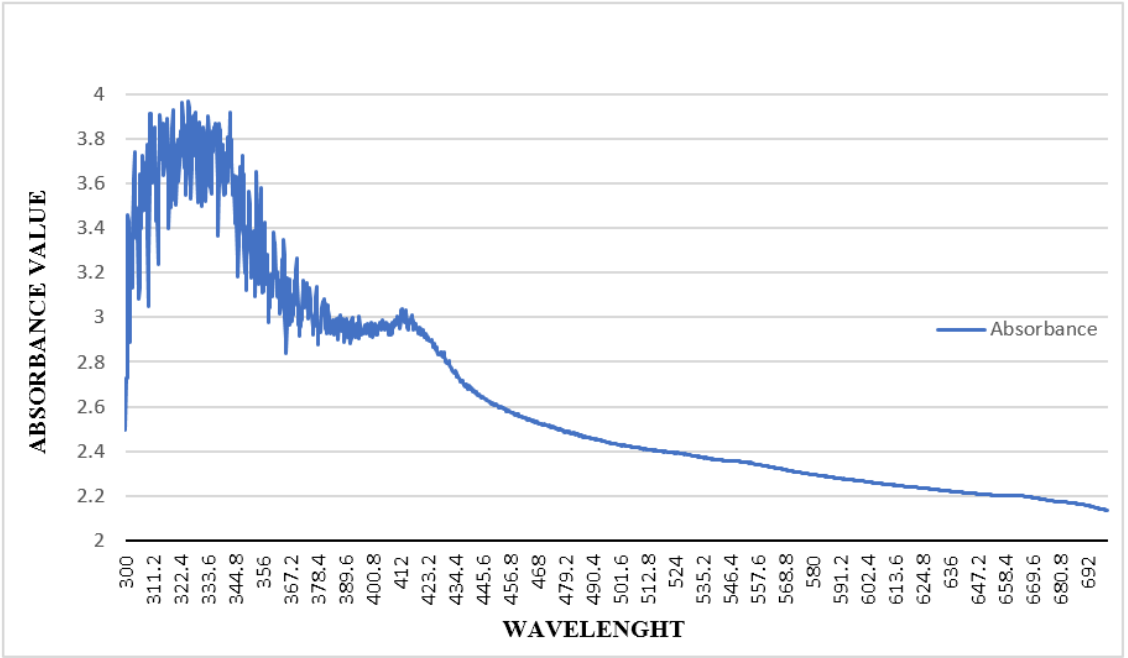
The isolated colonies on Nutrient Agar appeared off-white, opaque, moderately raised with irregular edges and a dry, rough surface-typical of *Bacillus* species. Gram staining revealed Gram-positive, rod-shaped cells arranged in pairs. The isolate was motile, endospore-forming, and showed positive results for catalase, oxidase, VP, citrate, starch, nitrate, casein, and gelatin hydrolysis, while negative for indole, methyl red, and urease. These morphological and biochemical characteristics confirmed the identity of the isolate as *Bacillus subtilis*.

B. Conformation of Biosynthesised CNPs

The formation of nanoparticles was visually confirmed by a distinct colour change. The control flask (without chitosan) retained its original light-yellow colour, whereas the experimental flask containing *Bacillus subtilis* and chitosan showed a pale brownish colour, indicating successful nanoparticle synthesis. After incubation, the nanoparticles were collected by centrifugation, and the pellet containing the chitosan nanoparticles was harvested (Figure 1).

Figure 1 Chitosan Nanoparticle Synthesis

Figure 2 Effect of Chitosan Nanoparticles on Dye Degradation of Textile Effluent



Graph 1 UV - Vis Spectroscopy Analysis of Chitosan Nanoparticles

C. UV-Vis Spectroscopy Analysis of CNPs

UV-Vis spectroscopy confirmed the formation of chitosan nanoparticles, with absorbance recorded in the 300–700 nm range. A peak at 325.6 nm (Abs = 3.9661) indicates nanoparticle presence, with secondary peaks at 323.0 nm (Abs = 3.9613) and 319.2 nm (Abs = 3.9326) confirming consistency (Graph 1). The high absorbance values suggest a significant nanoparticle yield, demonstrating the efficiency of biosynthesis using *Bacillus subtilis*.

D. Physico - Chemical Properties of Textile Effluent Water

The textile effluent exhibited a dark reddish-brown colour, indicating the presence of dye residues and other organic contaminants. The pH was recorded at 9.0, suggesting an alkaline nature, which is common in textile wastewater due to the use of dyes, detergents, and other processing chemicals. The temperature of the effluent was 32°C, which could influence microbial activity and chemical reactions within the wastewater. The dissolved oxygen (DO) level was measured at 4.8 mg/L, indicating moderate oxygen availability, though it may still be insufficient for sustaining aquatic life in receiving water bodies. Additionally, the optical density (OD) at 550 nm was 1.2, reflecting a high concentration of suspended particles and dye molecules, contributing to the effluent's turbidity and colouration.

E. Effect of CNPs on the Textile Effluent Water Treatment.

The degradation of textile effluent using chitosan nanoparticles (CNPs) was assessed over 72 hours at varying concentrations (1 mL, 2 mL, and 3 mL) of CNPs. The control flask (no CNPs). The initial OD value before treatment was recorded as 1.20. After 24 hours of treatment, the OD of the control flask was 1.18, while the flasks with CNPs showed reductions in OD Flask 1 (1 mL CNPs) had an OD of 1.06, Flask 2 (2 mL CNPs) showed 0.94, and Flask 3 (3 mL CNPs) had an OD of 0.81. The decolourization efficiency at 24 hours was 1.7% for the control, with Flask 1, Flask 2, and Flask 3 showing 11.7%, 21.7%, and 32.5% respectively (Figure 2, Table 1).

After 48 hours, the OD values further decreased, with Flask 1 showing 0.88, Flask 2 showing 0.71, and Flask 3 showing 0.54, while the control flask remained at 1.16. The decolourization efficiency increased to 3.3% for the control, 26.7% for Flask 1, 40.8% for Flask 2, and 55.0% for Flask 3. After 72 hours, the control flask's OD decreased slightly to 1.13, indicating minimal change, while the CNP-treated flasks showed more significant reductions, with Flask 1 at 0.750, Flask 2 at 0.550, and Flask 3 at 0.444. The decolourization efficiency at 72 hours was 5.7% for the control, 37.5% for Flask 1, 54.2% for Flask 2, and 63.0% for Flask 3, indicating that higher concentrations of CNPs resulted in more effective dye degradation (Graph 2 ,Table 2 ,Table 3).

In terms of other parameters, the colour of the effluent in the control remained dark reddish-brown, while the CNP-treated flasks showed progressive lightening light brown in Flask 1, light yellowish-brown in Flask 2, and pale yellow in Flask 3. The pH of the effluent decreased with increasing concentrations of CNPs, reaching 8.2 in Flask 1, 7.8 in Flask 2, and 7.4 in Flask 3. The temperature of the effluent also slightly decreased, with Flask 1 at 30°C, Flask 2 at 28°C, and Flask 3 at 27°C. Dissolved oxygen levels were improved in the CNP-treated flasks, with Flask 1 at 5.4 mg/L, Flask 2 at 5.8 mg/L, and Flask 3 at 6.2 mg/L, compared to 4.8 mg/L in the control.

Time (hours)	Control (Flask 4)	Flask 1 (1 mL CNP)	Flask 2 (2 mL CNP)	Flask 3 (3 mL CNP)
OD (Initial)	1.20	1.20	1.20	1.20
24 h OD	1.18	1.06	0.94	0.81
48 h OD	1.16	0.88	0.71	0.54
72 h OD	1.13	0.75	0.55	0.44

Table 1 Optical Density (OD) Reduction at 550 nm During Dye Degradation

Time (hours)	Control (Flask 4)	Flask 1 (1 mL CNP)	Flask 2 (2 mL CNP)	Flask 3 (3 mL CNP)
Decolourization (24 h, %)	1.7%	11.7%	21.7%	32.5%
Decolourization (48 h, %)	3.3%	26.7%	40.8%	55.0%
Decolourization (72 h, %)	5.7%	37.5%	54.2%	63.0%

Table 2 Dye Decolourization Efficiency (%) at Different Time Intervals

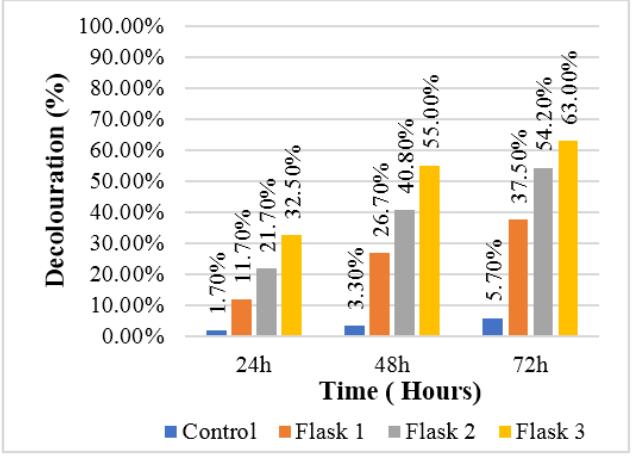
Parameter	Untreated Effluent	Control (No CNPs)	Flask 1 (1 mL CNPs)	Flask 2 (2 mL CNPs)	Flask 3 (3 mL CNPs)
Colour	Dark reddish-brown	Dark reddish-brown	Light brown	Light yellowish-brown	Pale yellow
pH	9.0	9.0	8.2	7.8	7.4
Temperature(°C)	32	32	30	28	27
Dissolved Oxygen (DO) (mg/L)	4.8	4.8	5.4	5.8	6.2
Optical Density (OD) at 550 nm	1.20	1.20	0.75	0.59	0.44
Decolourization Efficiency (%) (After 72 hours)	-	5.7%	37.5%	54.2%	63.0%

Table 3 Effect of Chitosan Nanoparticles on Physico-Chemical Properties and Dye Degradation of Textile Effluent After 72 Hours

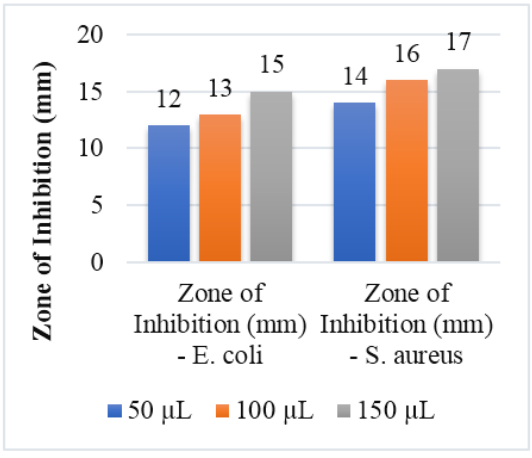
F. Antimicrobial Activity of CNPs

The antibacterial activity of chitosan nanoparticles synthesized using Bacillus subtilis was assessed against *Escherichia coli* and *Staphylococcus aureus* using the agar well diffusion method. After 24 hours of incubation, the inhibition zones for E. coli were 12 mm, 13 mm, and 15 mm for 50 µL, 100 µL, and 150 µL of nanoparticles, respectively. For *S. aureus*, the inhibition zones were 14 mm, 16 mm, and 17 mm for the same concentrations. (Figure 3) These results indicate that *S. aureus* exhibited greater sensitivity to chitosan nanoparticles than *E. coli*, as evidenced by the larger inhibition zones (Graph 3). The increasing inhibition zone with higher nanoparticle concentrations suggests a dose-dependent antibacterial effect.

Figure 3 Zone of Inhibition by Chitosan



Graph 2 Decolorization of Textile Effluent Using Chitosan



Graph 3 Zone of Inhibition by Chitosan

IV. CONCLUSION

The study confirms the successful isolation of *Bacillus subtilis* and its effective use in the biosynthesis of chitosan nanoparticles. UV–Vis analysis validated nanoparticle formation with a prominent peak at 325.6 nm. The synthesized CNPs significantly enhanced textile effluent decolourization and improved physico-chemical properties. Antibacterial assays demonstrated strong, dose-dependent inhibition against *E. coli* and *S. aureus*. These findings highlight the dual potential of biosynthesized CNPs in wastewater treatment and antimicrobial applications.

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