

DENITRIFICATION OF NITROGENOUS COMPOUNDS IN INDUSTRIAL WASTEWATER USING *Bacillus Tequilensis*

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ABSTRACT: An ideal wastewater treatment process should be simple, cost-effective, and capable of achieving high removal efficiency. In recent years, heterotrophic nitrification and aerobic denitrification (HN-AD) have gained attention as a promising approach for removing nitrogenous compounds such as ammonium nitrogen ($\text{NH}_4^+\text{-N}$), nitrite nitrogen ($\text{NO}_2^-\text{-N}$), and nitrate nitrogen ($\text{NO}_3^-\text{-N}$). In this study, the potential of *Bacillus tequilensis* to perform HN-AD was evaluated using 1% v/v diluted wastewater with an initial ammonium nitrogen ($\text{NH}_4^+\text{-N}$) concentration of 65 mg/L. Batch experiments were conducted with varying glucose concentrations (0–4% w/v) and incubated overnight at 37°C. The treatment performance was monitored by measuring changes in ammonium, nitrite, nitrate, pH, and bacterial growth. Without glucose supplementation, the culture showed minimal ammonium removal and considerable nitrite accumulation, suggesting incomplete nitrogen conversion. However, when glucose was added at 3% and 4% (w/v), ammonium removal improved significantly (approximately 65–72%) and nitrite accumulation gradually decreased. While nitrate levels remained relatively stable at lower glucose concentrations, they decreased noticeably at the highest glucose concentration. This finding suggests that the increased carbon availability may influence the nitrogen transformation pathway and enhance overall removal performance. This result indicates that carbon availability significantly impacts nitrogen transformation by *Bacillus tequilensis* in high-ammonium wastewater environments.

KEY WORDS: *Bacillus tequilensis*, High-ammonium, Ammonium-N, Nitrite, and Nitrate.

1. INTRODUCTION

High concentrations of nitrogen compounds in water bodies, such as ammonium nitrogen ($\text{NH}_4^+\text{-N}$), nitrite nitrogen ($\text{NO}_2^-\text{-N}$), and nitrate nitrogen ($\text{NO}_3^-\text{-N}$), can seriously harm aquatic ecosystems [1]. These pollutants often enter rivers and lakes due to insufficient or improper treatment of wastewater from domestic sewage, industrial activities, and landfill leachate. When released into the environment, excessive nitrogen can trigger eutrophication, leading to algal blooms, oxygen depletion, and the disruption of the natural ecological balance [2]. For this reason, effective treatment of nitrogen-rich wastewater is essential to safeguard aquatic life and protect human health, as clean water remains a critical resource for both environmental sustainability and community well-being.

One common method used in wastewater treatment is bioremediation. It is considered environmentally friendly, cost-effective, and easy to apply [3,4]. In conventional biological treatment systems, microorganisms play an important role in removing nitrogen compounds. Autotrophic nitrifying bacteria convert ammonium into nitrate through a process called nitrification (N). Then, heterotrophic denitrifying bacteria convert the nitrate into nitrogen gas through denitrification (D), allowing it to be safely released into the environment.

The conventional nitrification–denitrification (ND) process can be impractical because it requires two separate steps under strictly controlled aerobic and anaerobic conditions. Maintaining these conditions often leads to higher operating costs and makes the system more complex and time-consuming to manage. As an alternative, heterotrophic nitrification–aerobic denitrification (HN-AD) microorganisms offer a more efficient solution [5]. These microorganisms can carry out simultaneous nitrification and denitrification (SND) within a single system. Previous studies have shown that certain SND microorganisms can effectively remove nitrogen and reduce chemical oxygen demand (COD). In addition, the acid–base imbalance generated during nitrification can be naturally compensated for through concurrent denitrification. Overall, the SND process has the potential to simplify wastewater treatment operations while reducing operating costs, energy consumption, and carbon emissions [5,6]. However, this process requires a higher organic carbon source and an aeration rate than conventional biological denitrification [7].

Various microorganisms capable of degrading ammonium nitrogen have been reported in previous studies. For instance, *Klebsiella pneumoniae* TN-1 achieved 83.25% removal of ammonium nitrogen from an initial concentration of 300 mg/L after one week of incubation [8]. Similarly, *Sphingomonas* sp. strain LPN080 reduced ammonium nitrogen levels in wastewater from 8 mg/L to 0.3 mg/L within 48 hours [8]. *Pseudomonas chengduensis* BF6 demonstrated even higher efficiency, removing 97% of ammonium nitrogen (initial concentration of 472 mg/L) within 24 hours [8]. In addition, *Phialemoniopsis curvata* TWCC 58054s almost completely degraded 50 mg/L of ammonium nitrogen after seven days of incubation [9]. These studies collectively highlight the effectiveness of microbial nitrification in converting $\text{NH}_4^+\text{-N}$ in wastewater into nitrite and nitrate, offering a more sustainable approach with minimal formation of secondary pollutants.

Zhang et al. (2025) [10] reported the denitrification performance of 120 *Bacillus* isolates, where eight strains showed notable denitrification capabilities in nitrogen-containing wastewater. *Bacillus subtilis* B24 was identified to exhibit the highest denitrification efficiency, achieving removal rates of 92% for $\text{NH}_4^+\text{-N}$, 62% for $\text{NO}_2^-\text{-N}$, 68% for $\text{NO}_3^-\text{-N}$, and 30% for total nitrogen in simulated wastewater. Furthermore, Wang et al. (2023) [3] reported that *Bacillus tequilensis* A2, isolated from aquaculture wastewater, could degrade high concentrations of ammonium nitrogen. Their study demonstrated that this strain holds strong potential for wastewater treatment applications, particularly for systems with elevated ammonium and nitrite levels. Notably, it achieved a 95% ammonium nitrogen removal rate when incubated at 42°C, with an initial pH of 7 and a 5% inoculum volume [3]. Overall, these findings reinforce the significant potential of microbial-based strategies, particularly *Bacillus* sp., for efficient and sustainable nitrogen removal in wastewater treatment systems. *Bacillus* sp. are also recognized as safe, eco-friendly, highly competent and environmentally adaptable strains that are beneficial for improving water quality [10].

Previous studies have shown that *Bacillus* sp. have the potential to degrade pollutants in wastewater. However, evaluating their performance in reducing ammonium-N, nitrite, and nitrate concentrations under specific operational conditions, particularly carbon availability, is necessary. Thus, this study investigates the growth of *B. tequilensis* in ammonium-rich wastewater under various carbon concentrations, as well as its capacity to reduce these nitrogenous compounds.

2. MATERIAL AND METHODOLOGY

2.1. Wastewater Sample

A wastewater sample with high ammonium concentrations was collected from a local industry in Malacca, Malaysia.

2.2. Bacteria and culture medium

The bacterial culture used in this study consists of the *B. tequilensis* strain. The TS Broth (g/l) is a mixture of 17.0 g casein peptone, 3.0 g soymeal peptone, 2.5 g D(+) glucose monohydrate, 5.0 g NaCl, and 2.5 g di-potassium hydrogen phosphate. The LB Agar (g/l) consists of 15.0 g tryptone, 5.0 g soya peptone, 5.0 g NaCl, 15.0 g agar, with a pH of 7.3 ± 0.2 .

2.3. Chemicals and Reagents

All purchased chemicals were used as they were received. The following Bendosen chemicals were used: glucose (dextrose anhydrous) powder, di-potassium hydrogen phosphate, potassium dihydrogen phosphate (anhydrous), and ferrous sulfate heptahydrate. Magnesium sulfate heptahydrate (Chemiz), sodium chloride (Merck), calcium chloride (System), and ethanol (System) were also used. Reagents for ammonium, nitrite, nitrate, and COD analysis were purchased from HACH (Malaysia).

2.4. Inoculum Preparation

B. tequilensis was inoculated into 10 ml of the sterilized TS media. The inoculated medium was incubated overnight at 37°C in an incubator shaker with a constant rotation speed of 150 rpm. All procedures were carried out under a laminar flow hood and under aseptic conditions.

2.4.1. Aerobic Fermentation of *B. tequilensis* in Shake Flask

The overnight culture of *B. tequilensis* was used as the inoculum for the experiment. Initially, the 3% (v/v) overnight culture of *B. tequilensis* was inoculated into 200 ml of tryptic soy (TS) medium without glucose in a 500 ml shake flask. The medium was supplemented with 100 ml of 1% (v/v) diluted wastewater. The same experiment was repeated with different glucose concentrations of 1, 2, 3, and 4% w/v. Incubation was carried out in an incubator shaker at 37 °C and 150 rpm for 24 hours. Optical density (OD) at 600 nm was measured every 2 hours for the first 12 hours and again at 24 hours to monitor bacterial growth. This experiment was repeated three times.

2.4.2. Assessment of ammonium, nitrite, nitrate and COD

The concentration of ammonium, nitrite, nitrate, and COD was determined using the DR 2800 instrument, with the Nessler method, NitriVer 2 reagent, NitraVer X nitrate reagent, and COD Digestion HR kit, respectively.

3. RESULT AND DISCUSSION

Fig. 1 illustrates the OD₆₀₀ growth profile of *B. tequilensis* in 1% diluted wastewater with an initial ammonium-N concentration of 65 mg/l after 24 hours of incubation at 37°C. Fig. 1 shows a clear difference in biomass accumulation under varying glucose concentrations (1–4% w/v). In general, both the growth rate and the overall biomass production of *B. tequilensis* were strongly influenced by carbon availability. Higher glucose concentrations supported greater bacterial growth, indicating that carbon concentration plays a key role in regulating the culture's metabolic activity.

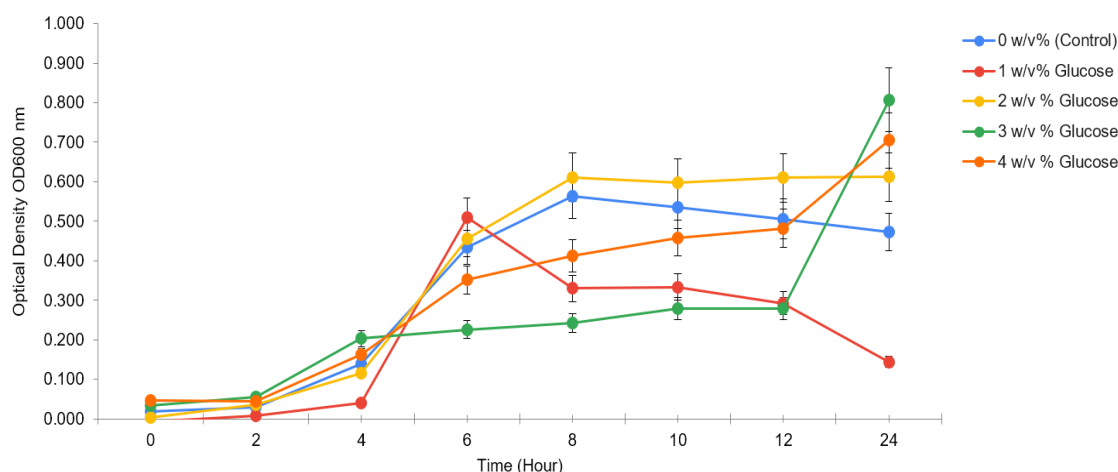


Fig. 1. Overview of *B. tequilensis* growth over time for different concentrations of glucose.

As shown in Fig. 1, during the first 6 hours of incubation, the OD₆₀₀ increased rapidly, particularly in cultures supplemented with 2–4% (w/v) glucose, reaching an early biomass concentration of approximately 0.60. This suggests that sufficient carbon availability accelerates initial cell division and promotes entry into the exponential growth phase. In contrast, limited early growth was observed in cultures supplied with 1% (w/v) glucose, indicating that insufficient carbon may restrict the transition into the exponential phase.

During the subsequent 6 hours (mid-growth phase), cultures supplemented with 2% and 4% (w/v) glucose maintained relatively stable OD values. This stability indicates that adequate nutrient availability sustained biomass without imposing metabolic stress. The control culture without glucose remained relatively stable, due to the gradual consumption of residual nutrients present in the medium. Conversely, the culture supplemented with 1% (w/v) glucose showed a significant decline after 6 hours of incubation, possibly due to nutrient depletion and the onset of the stationary or decline phase.

The highest biomass levels were achieved in cultures supplemented with 3% and 4% (w/v) glucose, reaching approximately 0.85 and 0.70, respectively. This observation suggests that higher carbon availability can prolong active cell growth by preventing early nutrient limitation and sustaining metabolic activity.

Overall, these results clearly demonstrate the impact of glucose concentration on growth rate, nutrient utilization, and biomass yield of *B. tequilensis*. The findings offer useful guidance for optimizing culture conditions, highlighting carbon availability as a key factor in enhancing bacterial growth performance.

Nitrogen transformation by *B. tequilensis* in 1% (v/v) diluted wastewater, with an initial ammonium-N concentration of 65 mg/l, was evaluated by measuring ammonium nitrogen ($\text{NH}_4^+\text{-N}$), nitrite nitrogen ($\text{NO}_2^-\text{-N}$), and nitrate nitrogen ($\text{NO}_3^-\text{-N}$) at different glucose concentrations (1–4% w/v) (Table 1). In the absence of glucose (control system), ammonium concentration decreased from 65 mg/L to 46 ± 3.5 mg/l, corresponding to a 29% removal efficiency. However, this reduction was accompanied by substantial accumulation of nitrite, suggesting incomplete nitrogen transformation. Although the original wastewater had an initial COD of >1500 mg/l, dilution reduced the availability of biodegradable organic carbon. This has limited the heterotrophic nitrogen consumption and disrupted balanced nitrogen conversion.

Table 1: Results of ammonium nitrogen ($\text{NH}_4^+\text{-N}$), nitrite nitrogen ($\text{NO}_2^-\text{-N}$), nitrate nitrogen ($\text{NO}_3^-\text{-N}$) concentrations and pH at different glucose concentrations (1–4% w/v)

Glucose Concentration [w/v%]	Ammonium [mg/l]	Nitrite [mg/l]	Nitrate [mg/l]	pH
0 (Control)	46 ± 3.5	94 ± 29	11.1 ± 0.7	8
1	38 ± 8.5	79 ± 38	10.7 ± 0.65	5.8
2	43 ± 2	66 ± 16.5	10.9 ± 1.7	5.7
3	23 ± 3	2	10.4 ± 3.35	3.3
4	18 ± 5	3 ± 0.5	3.7 ± 0.95	4.8

It is observed that ammonium-N removal improved under all glucose-supplemented conditions compared to the control. The ammonium-N concentration decreased to 38 ± 8.5 mg/l and 43 ± 2 mg/l in cultures supplemented with 1% and 2% (w/v) glucose, respectively, indicating enhanced nitrogen uptake. These results suggest that the additional carbon source was utilized by *B. tequilensis*, a heterotrophic bacterium, to support growth and promote the assimilation of ammonium into biomass. Similar findings have been reported in which *Bacillus* species preferentially assimilated ammonium under carbon-sufficient conditions during short incubation periods [11, 12]. Greater ammonium-N removal was achieved at higher glucose concentrations (3% and 4% w/v), with residual ammonium levels of 23 ± 3 mg/l and 18 ± 5 mg/l, respectively. These correspond to removal efficiencies of approximately 65–72%, indicating that higher C/N ratios favor heterotrophic nitrogen utilization. Comparable trends have been reported in wastewater treatment studies using *Bacillus* species, in which increased carbon availability enhanced ammonium removal via assimilation and other heterotrophic pathways [12].

Nitrite concentrations decreased noticeably as glucose levels increased. Under control conditions, substantial nitrite accumulation was observed, indicating incomplete nitrogen conversion. In contrast, at glucose concentrations of 3% to 4% (w/v), almost complete nitrite removal was achieved, suggesting that glucose supplementation effectively alleviated nitrite accumulation. Similar observations have been reported in previous studies, in which sufficient organic carbon facilitated the rapid transformation or utilization of nitrogen intermediates, thereby minimizing nitrite buildup, particularly in heterotrophic nitrogen removal systems. However, a comprehensive discussion of the nitrogen removal pathways cannot be fully established due to the limited dataset and short incubation period in this study.

A decrease in pH was observed in cultures supplemented with glucose, indicating active heterotrophic metabolism. This acidification is likely attributed to the production of organic acids during glucose metabolism. Such conditions may suppress autotrophic nitrification

activity while exerting minimal inhibitory effects on *Bacillus* species, potentially promoting a shift in nitrogen transformation toward heterotrophic pathways.

Overall, this study demonstrates that at an initial ammonium-N concentration of 65 mg/L, both ammonium removal efficiency and the reduction of nitrite accumulation in *B. tequilensis*-inoculated diluted high-COD wastewater were strongly influenced by glucose supplementation. Nevertheless, further investigations with longer incubation periods, total nitrogen analysis, and measurements of gaseous nitrogen are necessary to gain a deeper understanding of the dominant nitrogen removal mechanisms.

4. CONCLUSION

This study demonstrates that *B. tequilensis* transforms nitrogen into ammonium nitrogen ($\text{NH}_4^+\text{-N}$), nitrite nitrogen ($\text{NO}_2^-\text{-N}$), and nitrate nitrogen ($\text{NO}_3^-\text{-N}$) in 1% diluted wastewater with an initial ammonium-N concentration of 65 mg/L, and that this transformation is strongly influenced by carbon availability. The results show that glucose supplementation affected ammonium removal efficiency, nitrite and nitrate concentration profiles, and pH during 24 hours of incubation. This study can be a basis for predicting the performance of certain strains in treating real wastewater. Further investigation involving longer incubation periods, comprehensive nitrogen analysis, and carbon-to-nitrogen ratio (C/N) analysis is necessary to understand the primary nitrogen removal mechanisms.

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Generative AI Statement:

During the preparation of this manuscript, AI-assisted tools, including ChatGPT, were used to improve clarity, readability, and grammatical accuracy. All AI-generated content was critically reviewed, validated, and edited by the authors. The authors assume full responsibility for the integrity and accuracy of the final published work.

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