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STUDY ON BIOLOGICAL CHARACTERISTICS OF AMMONIA OXIDIZING BACTERIA ISOLATED FROM WASTEWATER SLUDGE AT SOME WOOD PROCESSING FACILITIES IN VIETNAM

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Abstract

Aim. This study investigates the biological characteristics of ammonia-oxidizing bacteria (AOB) isolated from wastewater sludge collected at several wood processing facilities in Vietnam.

Methodology. To achieve the objectives of the study, composite sludge samples were collected from wastewater treatment units and processed within 6 hours to preserve microbial integrity. Ammonium-oxidizing bacteria were isolated using serial dilution and selective AMS medium, followed by screening for ammonium removal capability. Physiological characteristics of the isolates were evaluated under varying pH, temperature, and ammonium concentrations. Molecular identification was performed by PCR amplification and sequencing of the 16S rRNA gene. The combined use of microbiological and molecular techniques provided a comprehensive understanding of the diversity and functional traits of ammonium-oxidizing bacteria in sludge.

Results. From six sludge samples collected at wood processing facilities in Yen Bai, Thanh Hoa, and Dong Nai provinces, a total of eleven bacterial strains with ammonium-oxidizing capabilities were isolated. Dong Nai samples yielded the highest number of isolates, suggesting favorable conditions for ammonia-oxidizing bacteria in this region. Among the isolates, three strains – *Bacillus subtilis* BT183.1.B1, *Bacillus velezensis* BT751.1.B2, and *Bacillus amyloliquefaciens* BT751.2.B1 – exhibited superior ammonium removal efficiency and were selected for detailed characterization. These strains showed optimal growth at neutral pH (7.0–7.5), temperatures between 35°C and 37°C, and ammonium concentrations up to 700 mg/L. Growth and activity decreased significantly at more extreme pH values, temperatures, and ammonium concentrations. The ability of these Bacillus strains to tolerate high ammonium levels and environmental variability highlights their potential for application in biological treatment of nitrogen-rich industrial wastewater, particularly in the wood processing sector.

Research implications. Vietnam ranks fifth globally, second in Asia, and first in Southeast Asia in wood and forest product exports. The rapid growth of the wood industry has brought substantial economic benefits but has also caused environmental challenges, particularly

pollution from wood processing wastewater containing high levels of ammonium – a pollutant difficult to treat biologically. These findings contribute to expanding the diversity of ammonium-oxidizing bacteria and highlight their potential application in treating ammonium-rich wastewater from wood processing industries.

Keywords: Ammonia-oxidizing bacteria, biological characteristics, environmental pollution, wood processing wastewater, *Bacillus*

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ИССЛЕДОВАНИЕ БИОЛОГИЧЕСКИХ ХАРАКТЕРИСТИК АММОНИЙ-ОКИСЛЯЮЩИХ БАКТЕРИЙ, ВЫДЕЛЕННЫХ ИЗ ОСАДКА СТОЧНЫХ ВОД НЕКОТОРЫХ ДЕРЕВООБРАБАТЫВАЮЩИХ ПРЕДПРИЯТИЙ ВЬЕТНАМА

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Аннотация
Цель. Данное исследование посвящено изучению биологических характеристик аммоний-окисляющих бактерий (АОБ), выделенных из осадка сточных вод, собранных на нескольких деревообрабатывающих предприятиях Вьетнама.
Процедура и методы. Для достижения целей исследования были отобраны объединённые пробы осадка из очистных сооружений и обработаны в течение 6 часов для сохранения целостности микрофлоры. Аммоний-окисляющие бактерии выделяли методом серийных разведений на селективной питательной среде AMS с последующим скринингом на способность удалять аммоний. Физиологические характеристики изолятов оценивали при различных значениях pH, температуры и концентрации аммония. Молекулярная идентификация проводилась с помощью ПЦР-амплификации и секвенирования гена 16S рРНК. Комбинированное использование микробиологиче-

ских и молекулярных методов позволило получить комплексное представление о разнообразии и функциональных признаках аммоний-окисляющих бактерий в осадке.
Результаты. Из 6 проб осадка, собранных на деревообрабатывающих предприятиях в провинциях Йенбай, Тханьхоа и Донгнай, было выделено в общей сложности одиннадцать штаммов бактерий, обладающих способностью окислять аммоний. Пробы из Донгная дали наибольшее количество изолятов, что свидетельствует о благоприятных условиях для аммоний-окисляющих бактерий в этом регионе. Среди выделенных штаммов три − Bacillus subtilis BT183.1.B1, Bacillus velezensis BT751.1.B2 и Bacillus amyloliquefaciens BT751.2.B1 − продемонстрировали наивысшую эффективность удаления аммония и были отобраны для детальной характеристики. Эти штаммы показали оптимальный рост при нейтральном pH (7,0−7,5), температуре от 35°C до 37°C и концентрации аммония до 700 мг/л. Рост и активность значительно снижались при более экстремальных значениях pH, температуры и концентрации аммония. Способность данных штаммов Bacillus переносить высокие уровни аммония и изменчивость условий окружающей среды подчёркивает их потенциал для применения в биологической очистке богатых азотом промышленных сточных вод, особенно в деревообрабатывающей отрасли.
Теоретическая и/или практическая значимость. Вьетнам занимает пятое место в мире, второе в Азии и первое в Юго-Восточной Азии по экспорту древесины и лесной продукции. Быстрый рост деревообрабатывающей отрасли принёс значительные экономические выгоды, но также создал серьёзные экологические проблемы, в частности, загрязнение от сточных вод деревообработки, содержащих высокие уровни аммония − загрязнителя, трудно поддающегося биологической очистке. Полученные результаты способствуют расширению знаний о разнообразии аммоний-окисляющих бактерий и подчёркивают их потенциальное применение для очистки богатых аммонием сточных вод деревообрабатывающей промышленности.

Ключевые слова: аммоний-окисляющие бактерии, биологические характеристики, загрязнение окружающей среды, сточные воды деревообрабатывающей промышленности, *Bacillus*

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INTRODUCTION

Vietnam is recognized as the fifth largest exporter of wood and forest products worldwide, ranking second in Asia and first in Southeast Asia [1]. The rapid expansion of the wood processing industry has significantly contributed to the national economy. However, this growth has also led to serious environmental challenges, particularly the generation of large volumes of wood processing wastewater containing high concentrations of ammonium (NH_4^+), a nitrogenous compound that poses considerable treatment difficulties due to its toxicity and persistence in aquatic ecosystems [2].

Ammonium in wastewater exists primarily in two forms: ammonia gas (NH_3) and ammonium ion (NH_4^+), both of which contribute to nitrogen pollution and eutrophication in receiving water bodies [3]. High ammonium levels can lead to dissolved oxygen depletion, algal blooms, and fish mortality, making its removal a priority in wastewater treatment strategies [4].

The biological removal of ammonium is a complex, multi-step process involving diverse microbial communities. Nitrification, the aerobic oxidation of ammonium to nitrite (NO_2^-) and subsequently nitrate (NO_3^-), is a critical first step facilitating further nitrogen removal via denitrification or anaerobic ammonium oxidation (anammox) processes [3;

4; 5]. These microbial transformations play an essential role in reducing nitrogen loads and mitigating environmental risks associated with nitrogen pollution.

Ammonia-oxidizing bacteria (AOB) such as *Nitrosomonas* and *Nitrosospira* are key players in the nitrification process. However, these bacteria are often difficult to isolate and cultivate due to their slow growth rates and sensitivity to environmental fluctuations such as pH, temperature, dissolved oxygen (DO), and chemical oxygen demand (COD) [5]. Autotrophic AOB are particularly vulnerable to competition from heterotrophic microorganisms, which often dominate in organic-rich wastewaters like those from wood processing plants [4].

In contrast, heterotrophic bacteria, particularly those of the genus *Bacillus*, have demonstrated robust ammonium oxidation capabilities. *Bacillus* species are known for their rapid growth, environmental resilience, and ability to metabolize ammonium even at high concentrations exceeding 1000 mg/L [6]. Several studies have reported that certain *Bacillus* strains can directly convert ammonium into free nitrogen gas, bypassing intermediate accumulation of nitrite or nitrate, offering significant advantages for wastewater treatment applications [7].

Given these characteristics, this study focuses on isolating and characterizing *Bacillus* strains from wastewater sludge at wood processing facilities in Vietnam. The aim is to elucidate their biological properties, including optimal growth conditions and ammonium oxidation capacity, to explore their potential for bioremediation of ammonium-rich industrial wastewater. This research contributes to expanding the diversity of known ammonium-oxidizing bacteria and provides a foundation for developing effective biological treatment technologies tailored to the wood processing industry.

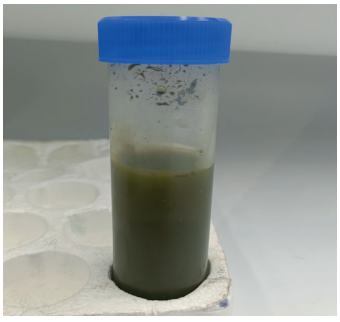
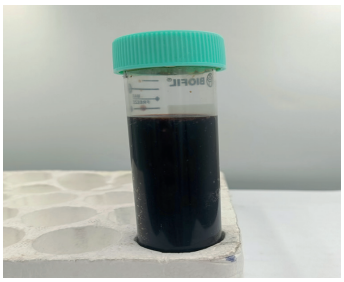
MATERIALS AND METHODS

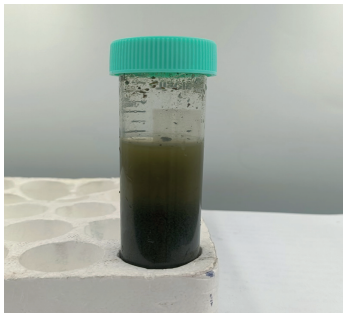
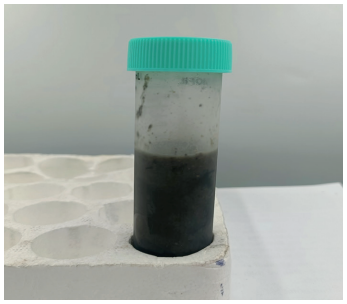
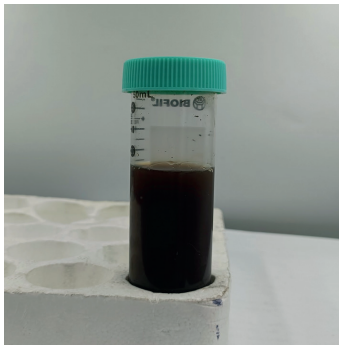
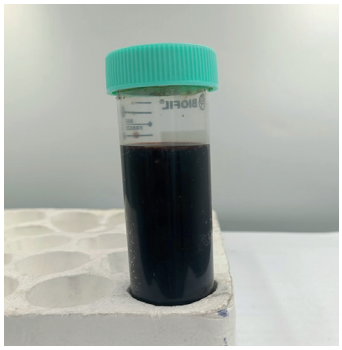
Materials

The research materials (selected samples for laboratory analysis) are presented in Table 1.

Table 1 / Таблица 1

List of samples / Список образцов

No.	Place	Code sample	Describe
01	Yen Bai province	BT183.1	 Blueish black
		BT183.2	 Black color, thick consistency

No.	Place	Code sample	Describe
02	Thanh Hoa province	BT111.1	 Blueish black
		BT111.2	 Dark brown
03	Dong Nai province	BT751.1	 Black color, thick consistency
		BT751.2	 Black color, thick consistency
Total		**6**	

Source: compiled by the authors

Methods

Sample Collection: Composite sludge samples were obtained using a sterile stainless-steel sludge sampler (depth 0–50 cm) at three different points within each treatment unit to ensure representative coverage. The samples were collected in sterile 1 L polypropylene containers, pre-labeled, and stored at 4°C in an insulated icebox during transportation to the laboratory. All samples were processed within

6 hours of collection to minimize microbial activity loss and compositional changes [8].

Isolation of Microorganisms from Sludge Samples: To isolate ammonium-oxidizing bacteria from sludge samples, a serial dilution and spread plate technique was employed using selective and enrichment media. All microbiological procedures were conducted under aseptic conditions in a laminar flow cabinet. Approximately 10 g of well-homogenized sludge was suspended in 90 mL of sterile 0.85% saline solution and vortexed vigorously for 10 minutes to dislodge microbial cells. The resulting suspension was allowed to settle for 5 minutes, and the supernatant was serially diluted (10^{-1} to 10^{-6}). Selective enrichment was performed using a modified ammonium mineral salts (AMS) medium, containing (per liter): $(NH_4)_2SO_4$ – 1.0 g; $MgSO_4.7H_2O$ – 0.2 g; $CaCl_2$ – 0.02 g; KH_2PO_4 – 1.0 g; Na_2HPO_4 – 1.0 g; $FeSO_4.7H_2O$ – 0.01 g; trace elements – 1 mL; pH adjusted to 7.2. The medium was supplemented with 0.5% glucose to support heterotrophic nitrifiers [9]. From each dilution, 0.1 mL was spread onto AMS agar plates and incubated at 30 ± 2 °C for 3–5 days. Colonies exhibiting distinct morphologies (mucoid, opaque, cream-white, round) were selected and purified by repeated streaking on fresh AMS plates [7; 9]. Preliminary screening for ammonium oxidation capability was performed by inoculating isolates into 50 mL of liquid AMS medium containing 100 mg/L NH_4^+-N. After incubation for 5 days at 30°C and 120 rpm, ammonium concentration was determined using the Nessler's reagent method, and positive isolates showing a significant decrease in NH_4^+ were retained for further study [7, 8]. Pure cultures were preserved in glycerol stocks (20%) at −80°C for long-term storage.

Determination of biological characteristics of bacterial strains: Effect of pH: Investigation in culture media with pH levels of 5, 6, 7, 8 and 9; Effect of temperature: Temperature levels of 5, 30, 37, 45 and 50 °C were investigated; Effect of ammonium concentration: the ammonium concentrations investigated were 100 mg/L, 300 mg/L, 500 mg/L, 700 mg/L, 1000 mg/L. Each experiment was repeated 3 times [10].

PCR Amplification and Sequencing of 16S rRNA Gene: The 16S rRNA gene of bacterial isolates was amplified using the universal primer pair 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGTTACCTTGTTACGACTT-3′). The PCR reaction mixture (25 μL) included 12.5 μL of 2Ч PCR Master Mix, 1 μL of each primer (10 μM), 1 μL of template DNA, and nuclease-free water. Thermocycling was performed with the following conditions: initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 90 s; and a final extension at 72°C for 10 min [11]. Amplification products were confirmed via 1.5% agarose gel electrophoresis stained with ethidium bromide. Target bands were purified using a commercial gel extraction kit according to the manufacturer's instructions and submitted for Sanger sequencing [12].

Sequence analysis: The sequencing results were compared to related data in Genbank by the BLAST search on NCBI.

RESULTS AND DISCUSSIONS

Isolation and identification results

From six wastewater sludge samples collected at wood processing facilities in Yen Bai, Thanh Hoa, and Dong Nai provinces, a total of 11 bacterial strains exhibiting ammonium oxidation activity were successfully isolated. The highest number of isolates (five strains) originated from Dong Nai, indicating a potentially higher microbial diversity or more favorable conditions for the proliferation of ammonia-oxidizing bacteria (AOB) in this region (Table 2).

The variation in isolate numbers among locations may be attributed to differences in the physicochemical characteristics of the sludge, such as ammonium concentration, pH, temperature, organic matter content, and other site-specific environmental factors. Previous studies have shown that the environmental context of sampling sites significantly influences the abundance and activity of specialized microbial groups such as AOB [1; 2].

Moreover, the higher number of isolates from Dong Nai may serve as a valuable basis for developing microbial technologies aimed at nitrogen removal in industrial wastewater treatment processes in this area.

Among the eleven isolates, three strains demonstrated superior ammonium metabo-

Table 2 / Таблица 2

Statistics of the number of strains isolated in the samples / Статистика количества штаммов, выделенных в образцах

Samples	BT183.1	BT183.2	BT111.1	BT111.2	BT751.1	BT751.2	**Total**
Strains	3	1	1	1	3	2	**11**

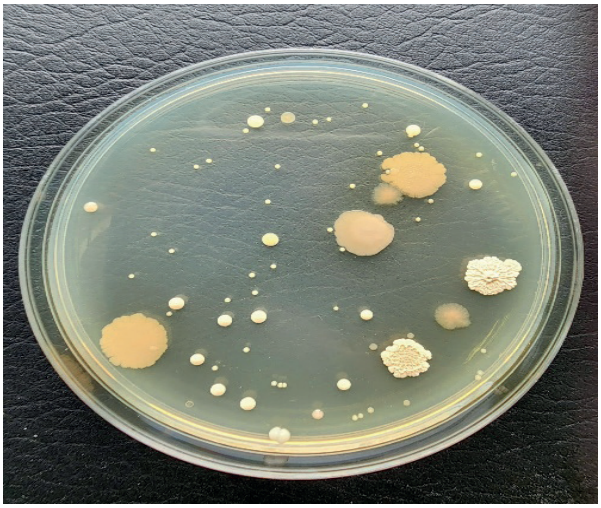


Fig. 1 / Рис. 1. Isolation results of sample BT175.1 / Результаты изоляции образцов BT175.1

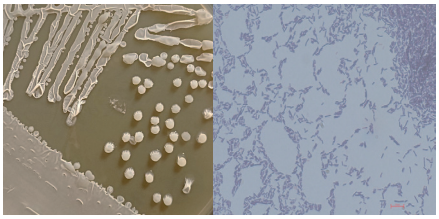
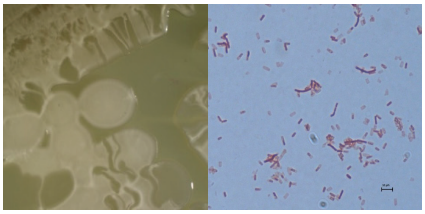
Source: compiled by the authors

lism efficiency and were selected for detailed characterization. Molecular identification based on 16S rRNA gene sequencing revealed that these strains shared hight sequence similarity with *Bacillus subtilis*, *Bacillus velezensis*, and *Bacillus amyloliquefaciens*, respectively. These strains were designated as *Bacillus subtilis* BT183.1.B1, *Bacillus velezensis* BT751.1.B2, and *Bacillus amyloliquefaciens* BT751.2.B1.

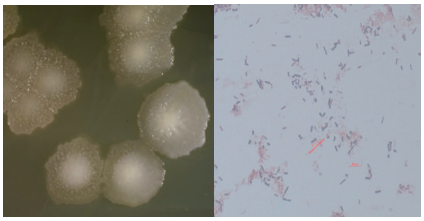
The identification of these *Bacillus* species is consistent with previous studies that underscore the genus's prominent role in ammonium oxidation across diverse wastewater environments, including both industrial and agricultural effluents. Their occurrence in sludge from wood processing wastewater highlights their adaptability to complex and potentially

Table 3 / Таблица 3

List of bacterial strains / Список штаммов бактерий

No.	**Strains**	**Species**	**Similarity level**	**Morphology**
1	BT183.1.B1	*Bacillus subtilis*	99,89% (KT236337)	 Colony: Colonies are circular with entire margins, slightly wrinkled or dry surface, opaque white to pale yellow in color. Cell: Rod-shaped, Gram-positive cells, capable of forming central or subterminal spores, usually non-motile or weakly motile.
2	BT751.1.B2	*Bacillus velezensis*	99% (NR116240)	 Colony: Colonies are irregular or rosette-shaped, heavily wrinkled surface, with undulate or serrated margins, cream to deep yellow color, slightly sticky and opaque. Cell: Rod-shaped, Gram-positive cells, spore-forming, and motile.

Окончание табл. 3

3	BT751.2.B1	*Bacillus amyloliquefaciens*	100% (NR041455)	 Colony: Colonies are round with irregular margins, dry surface, cream to light yellow in color, opaque. Clear hydrolysis zones may be visible on starch-containing media. Cell: Rod-shaped, Gram-positive cells, motile, and spore-forming.

Source: compiled by the authors

inhibitory conditions, such as elevated ammonium concentrations and high organic loads.

These results align with recent findings in Vietnam. For instance, Nguyen Thi Tham et al. [13] successfully isolated *Bacillus subtilis* and other heterotrophic nitrifying bacteria from aquaculture wastewater, reinforcing the prevalence and effectiveness of *Bacillus* species in ammonium removal across varied wastewater sources. Similarly, studies on nitrate-oxidizing bacteria in aquaculture systems in Thanh Hoa and Soc Trang provinces reported ammonium conversion efficiencies exceeding 95% under optimized conditions [14], further demonstrating the potential of indigenous *Bacillus* strains for practical application in local wastewater treatment systems [15].

Comparable results have been reported internationally. For example, Zhang et al. [16] isolated efficient ammonium-oxidizing *Bacillus* strains from municipal wastewater treatment plants in China, achieving removal rates above 90% under aerobic conditions. In another study, Yang et al. [17] highlighted the role of *Bacillus megaterium* in simultaneous nitrification and denitrification in high-ammonium synthetic wastewater. Likewise, Harshvardhan and Jha [18] demonstrated the capability of marine *Bacillus* spp. to degrade nitrogen-rich plastic-associated biofilms, underscoring the genus's environmental versatility.

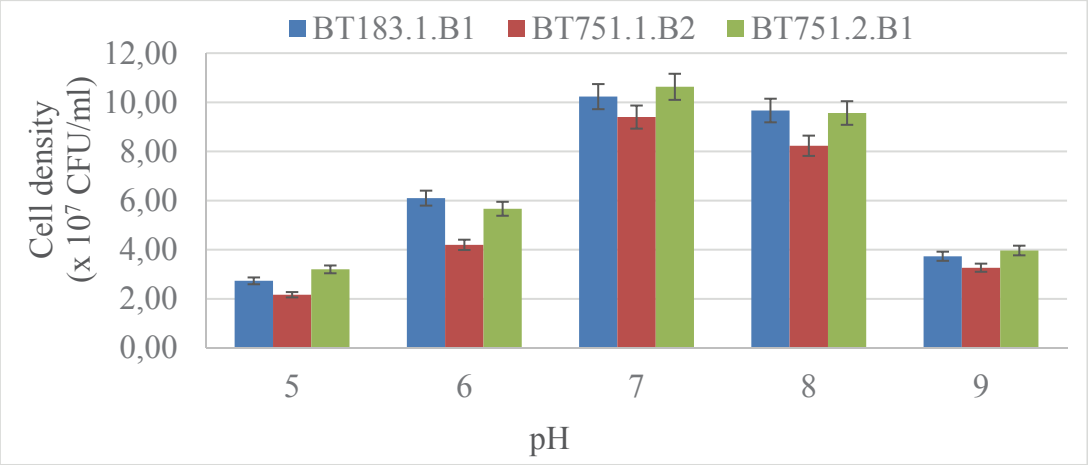
Effect of pH on growth of bacterial strains

The three *Bacillus* strains exhibited optimal growth in culture media with pH values ranging from 7.0 to 7.5, which corresponds closely to neutral conditions commonly found in natural and engineered wastewater treatment systems. Among them, *Bacillus amyloliquefaciens* BT751.2.B1 showed the highest growth rate at this pH range, suggesting a slightly better adaptation to neutral pH environments. Growth rates declined significantly at acidic (pH 5–6) and alkaline (pH 8–9) conditions, indicating sensitivity to pH extremes. This pH preference is consistent with the enzymatic activity of ammonia monooxygenase (AMO), the key enzyme catalyzing ammonium oxidation, which functions optimally near neutral pH. Maintaining pH within this optimal range is therefore critical for maximizing ammonium removal efficiency in bioreactors employing these strains.

These results are in line with international findings, such as those by Kim et al. [19], who demonstrated that *Bacillus* strains maintain robust ammonium oxidation activity at neutral pH and mesophilic temperatures, which are typical for many wastewater treatment systems globally. Furthermore, Bellucci and Curtis [20] highlighted the challenges of isolating autotrophic ammonia-oxidizing bacteria due to their slow growth and environmental sensitivity, whereas heterotrophic *Bacillus* species – like those isolated in this study –exhibit faster growth and greater adaptability. This advantage is especially relevant for practical applications in industrial wastewater treatment, where environmental conditions can fluctuate significantly.

Effect of temperature on growth of bacterial strains

Temperature is a crucial factor influencing microbial metabolism and enzyme kinetics. The three *Bacillus* strains demonstrated opti-



Pic. 2 / Рис. 2. Effect of pH on growth of bacterial strains / Влияние pH на рост бактериальных штаммов

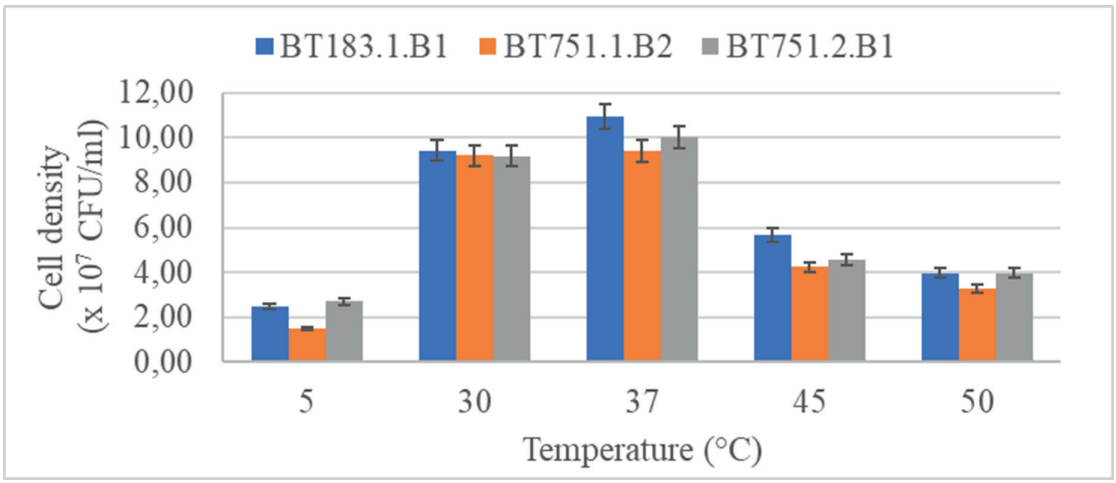
Source: compiled by the authors

mal growth at temperatures between 35°C and 37°C, with *Bacillus subtilis* BT183.1.B1 exhibiting the highest growth rate within this range. Growth was substantially inhibited at temperatures below 30°C and above 45°C, reflecting the mesophilic nature of these bacteria.

This temperature range aligns well with typical conditions in tropical wastewater treatment environments, including those in Vietnam, facilitating their practical application without the need for extensive temperature control [13]. The ability to maintain high ammonium oxidation rates at these temperatures enhances the feasibility of deploying these strains in large-scale treatment systems.

Similar observations have been reported by Zhang et al. [16], who found that indigenous *Bacillus* strains isolated from municipal wastewater systems in China exhibited optimal nitrification activity around 35 °C, with significant performance drops outside the 30–40 °C range. Likewise, Yang et al. [17] confirmed that *Bacillus megaterium* maintained simultaneous nitrification–denitrification capacity at mesophilic temperatures, further affirming the suitability of *Bacillus* species for mainstream treatment systems operating under mild thermal conditions.

In addition, Kim et al. [19] noted that while autotrophic nitrifiers often exhibit narrow optimal temperature ranges and decreased activity under fluctuating conditions, heterotrophic *Bacillus* strains display broader thermal tolerance and faster recovery from stress. This flex-



Pic. 3 / Рис. 3. Effect of temperature on growth of bacterial strains / Влияние температуры на рост штаммов бактерий

Source: compiled by the authors

ibility is particularly beneficial for decentralized or industrial treatment plants where environmental factors may be less controllable [8; 20].

Effect of ammonium concentration on growth of bacterial strains

The three *Bacillus* strains were tested for their capacity to metabolize ammonium at varying concentrations: 100, 300, 500, 700, and 1000 mg/L. All strains completely oxidized ammonium at concentrations up to 700 mg/L within five days of incubation, demonstrating strong tolerance and metabolic activity under high ammonium loads typical of wood processing wastewater. At 1000 mg/L ammonium, bacterial growth and ammonium oxidation rates declined markedly, indicating inhibitory effects likely due to ammonia toxicity or substrate inhibition. This finding is consistent with reports that high ammonium concentrations can disrupt cellular processes and enzyme function in nitrifying bacteria [13; 23].

The ability to efficiently oxidize ammonium at concentrations up to 700 mg/L positions these *Bacillus* strains as promising candidates for bioremediation of ammonium-rich industrial effluents. Their performance suggests potential for integration into biological treatment systems, either as pure cultures or as part of microbial consortia, to enhance nitrogen removal efficiency. This tolerance is particularly important for treating wastewater from wood processing and food industries, where ammonium concentrations often exceed the thresholds tolerated by traditional autotrophic nitrifiers [21; 22].

This outcome is consistent with both domestic and international studies. Vietnamese research has shown that locally sourced *Bacillus* strains can achieve high ammonium removal efficiency in both laboratory and field settings [13; 15]. For example, Pham et al. [15] reported efficient ammonium oxidation by indigenous *Bacillus* species up to 600–700 mg/L, with minimal performance loss. Internationally, Kim et al. [19] observed that selected *Bacillus* strains maintained nitrification activity even at concentrations exceeding 1000 mg/L, although metabolic inhibition was evident at extreme loads. Similarly, Zhang et al. [16] found that *Bacillus cereus* strains tolerated up to 800 mg/L ammonium without significant biomass loss, demonstrating strain-specific tolerance levels.

Such findings reinforce the value of using robust, heterotrophic ammonium-oxidizing bacteria like *Bacillus* in practical treatment applications, particularly under high-strength wastewater conditions where autotrophic nitrifiers underperform due to their lower resistance to ammonium toxicity and slower growth rates [24; 25].

CONCLUSION

This study successfully isolated eleven ammonia-oxidizing bacterial strains from wastewater sludge at wood processing facilities in Vietnam. Three *Bacillus* strains – *B. subtilis* BT183.1.B1, *B. velezensis* BT751.1.B2, and

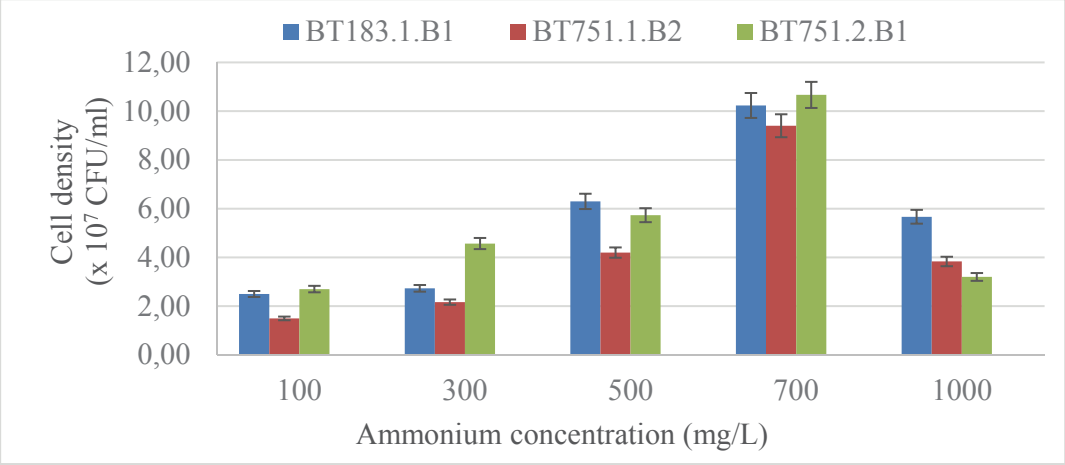


Рис. 4 / Рис. 4. Effect of ammonium concentration on growth of bacterial strains / Влияние концентрации аммония на рост бактериальных штаммов

Source: compiled by the authors

B. amyloliquefaciens BT751.2.B1 – demonstrated strong ammonium oxidation capacity under optimal conditions of pH 7–7.5 and 35–37°C. These strains completely metabolized ammonium at concentrations up to 700 mg/L, underscoring their potential for bioremediation applications in wood processing wastewater treatment.

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