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Nitrite metabolism of several bacterial strains isolated from abattoir and swine wastewater after biogas treatment

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Abstract. In nitrogen treatment with biological methods, nitrite metabolism is an intermediate process that facilitates other processes involving different bacteria strains. In this study, we isolated two nitrite-oxidising bacteria strains from abattoir wastewater and wastewater from biogas tanks of an industrial pig farm in Ha Tinh province. The bacteria strains grow, develop, and metabolise nitrite at pH 6–8 and 30–37 °C. The samples with the nitrite concentration up to 750 mg·L^{-1} were oxidised within four days of incubation, and the nitrite metabolism rate was proportional to the concentration of nitrite tested. Under severe conditions (salinity up to 3% NaCl, a low dissolved oxygen level of 0.1 mg·L^{-1}), the two isolated bacterial strains exhibited their effective growth and nitrite metabolism capacity. The results enrich the database of nitrite-oxidising bacteria and are prospective in wastewater treatment.

Keywords: nitrite, nitrite metabolism, swine wastewater, abattoir wastewater

1 Introduction

The treatment of nitrogen compounds, such as NH_4^+, NO_2^-, NO_3^-, in wastewater has great significance in protecting the environment as well as human health [1]. To remove nitrogen compounds effectively and sustainably, researchers mostly use biological methods [1, 2]. These methods are based on a process in which microorganisms convert nitrogen compounds into nitrogen gas (N_2) via nitrification and denitrification. In nitrification, ammonium-oxidising bacteria (AOB) oxidise ammonium to nitrite (ammonium oxidation), and then nitrite-oxidising bacteria (NOB) oxidise nitrite to nitrate (nitrite oxidation). In denitrification, nitrate is reduced back to nitrite, followed by nitrite reduction to nitrogen [1-3]. In other words, nitrite is an intermediate product of nitrification and denitrification, and the nitrate-to-nitrite conversion facilitates other processes taking place in the biological treatment of nitrogen. These processes involve different groups of autotrophic bacteria that use the energy released from nitrite metabolism to grow and develop [4]. Currently, numerous bacteria strains have been isolated and demonstrated their ability to metabolise nitrite. The most common bacteria are *Nitrobacter* [5-11], *Nitrospira* [12-17], *Nitrococcus* [18, 19], *Nitrospina*

[18, 19], *Candidatus Nitrotoga* [19], etc. However, in nature as well as in conventional applications in wastewater treatment plants, there are still some limitations, such as small bacterial growth and development rate due to the competition of NOB with other groups of microorganisms in wastewater [20], an insignificant cell division rate, high sensitivity to environmental conditions such as pH, temperature, sunlight, chemical oxygen demand (COD), and dissolved oxygen (DO) [21].

The nitrite metabolism process involves heterotrophic bacteria groups [22-24] with superior properties over other autotrophic-oxidising bacteria groups. They exhibit rapid growth and high development rate, quick cell doubling time, high competitiveness with other groups of bacteria in wastewater, the good adaptability to different environmental conditions such as pH, temperature, COD, and DO. Numerous studies elsewhere have reported on the nitrite oxidation capacity of heterotrophic bacteria, such as *Limosilactobacillus Plantarum* [25], *Pediococcus pentosaceus* [26], *Leuconostoc mesenteroides* [27], *Lactobacillus casei subsp. Rhamnosus* [28], *Lactobacillus fermentum* [28-33], and *Pseudomonas stuzeri* [34-36]. However, the number of relevant studies in Vietnam is moderate. Therefore, this study aims to isolate heterotrophic bacterial strains, identify their biological characteristics, and investigate nitrite metabolism. The findings help to confirm the diversity of the group of nitrite-oxidising bacteria and open their potential applications in the biological treatment of nitrogen in wastewater.

2 Materials and methods

2.1 Sampling

The wastewater samples were collected from Tan Giang concentrated slaughterhouse at Block 10, Tan Giang ward, Ha Tinh City (base coordinates X = 2028349 m; Y = 0543280 m) and from wastewater after biogas treatment at a pig farm in Ky Dong town, Ky Anh district, Ha Tinh province (base coordinates X = 2011792 m; Y = 0578757 m). Four litres of samples were collected and stored in specialised sterile plastic bags, then put in an insulated foam box containing dry ice, brought to the laboratory, and subjected to experiments within 36 hours after collection. The samples were shaken vigorously and filtered to remove dregs before testing.

2.2 Chemicals and instrument

All chemicals were sourced from Merck (Germany) and Hanna (Romania) with a purity greater than 99%. Nitrite concentration was determined with a nitrite portable photometer (Hanna, Model HI 96707, Rumania) with a range of 0–0.6 $mg{\cdot}L^{-1}$ and an error of 0.001 $mg{\cdot}L^{-1}$. Bacterial densities were obtained by directly counting from the Neubauer chamber (Hanna) of a microscope and controlling the water volume to obtain the required density for the experiment.

2.3 Methods

Bacterial culture and isolation

The working sample was prepared by diluting a stock with sterile distilled water 10^5 times, as follows: adding 5 mL of the stock into 45 mL of distilled water and thoroughly mixing (10×dilution); the dilution was conducted in the same manner until a 10^{-5} diluted sample was obtained. All dilution steps were carried out under sterile conditions in an incubator. The samples were then isolated in test tubes containing 10 mL of a mineral medium with 1 g $NaNO_2$, 1 g Na_2CO_3, 0.5 g NaCl, 0.5 g K_2HPO_4, 0.3 g $MgSO_4{\cdot}7H_2O$, 0.4 g $FeSO_4{\cdot}7H_2O$, and 2 g of low-melting agar 2%, and filled with distilled water until a volume of 1 L was obtained; pH was set around 8.3–8.8. The test tubes were kept at 37 °C in an incubator [37]. The colony formation was

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observed concurrently with nitrite oxidation through qualitative reactions with Griess reagents. Culture time was set for five days, and nitrite conversion was determined every 24 h of culture. In the case of a positive reaction, the reagent colour faded from pink to colourless when nitrite oxidation was completed. The culture tubes with positive reactions were selected for the following step of bacterial isolation.

The bacterial colonies with different shapes and colours in test tubes were subcultured separately in new test tubes until the shape and colour uniformity was achieved when the colonies were considered purely. In addition, the test tubes containing bacterial colonies were studied for their ability to oxidise nitrites during culture and isolation to eliminate those not oxidising nitrite.

Identification of bacteria species

The bacterial strains were investigated based on colony homogeneity on the isolated media, identified with the polymerase chain reaction amplification and sequencing with the 16S rRNA gene, and searched with the BLAST program. DNA of nitrite-oxidising bacteria was extracted by using the Macherey-Nagel kit (Fisher Scientific Company). The DNA samples were then purified with a Promega kit before amplification in a T100TM thermal cycler (Bio-Rad Laboratories) with a 27f/1492r primer set. The amplified DNA samples were checked for purity by using the Wide Mini-Sub Cell GT Horizontal Electrophoresis System (Bio-Rad Laboratories, USA). The electrophoresis samples were imaged and analysed with the omniDOC Gel Documentation System from the UK. Finally, the DNAs were sequenced on an automated Sanger Sequencing DNA Analyzer from the USA.

Cultural characteristics of isolated bacterial strains

The cultural characteristics include growth, development, and nitrite metabolism, and they were studied under the following conditions.

- pH ranging from 5 to 8.5 and adjusted with a 0.5 M NaOH solution or a 0.5 M HCl solution.
- Temperature ranging from 5 to 50 °C. The culture vessels were kept at 30, 37, 45, and 50 °C in an incubator; the control was stored at 5 °C in a refrigerator.
- Dissolved oxygen concentration was set at 0.1, 4.5, and 7.0 $mg \cdot L^{-1}$. The values were adjusted with CO_2 or the air.
- NaCl concentration was set at 1, 3, and 5%.
- Initial nitrite concentration was set at 100, 500, and 750 $mg \cdot L^{-1}$.

Each experiment was replicated three times. The culture medium used an initial bacterial culture density of 10^6 $CFU \cdot mL^{-1}$. When investigating the influence of each factor, control experiments were conducted simultaneously, including medium flasks containing nitrite and without bacterial strains (ĐC1) or containing bacterial isolates that were sterilised at 121°C and 1 atm for 15 min before adding to the medium (ĐC2). The nitrite-metabolising capacity of the isolates was evaluated based on the decrease in the concentration of the tested substances. Note that the byproducts of metabolisms were not considered.

3 Results and discussion

3.1 Bacterial isolation and identification

From the concentrated slaughterhouse wastewater samples and post-biogas wastewater samples from commercial pig farms, we isolated eight pure bacterial strains. Two of the strains could metabolise nitrite. The gram staining results

showed that one strain was Gram-positive bacteria (purple), and the other was Gram-negative bacteria (pink) (Fig. 1). Two stains were identified, belonging to *Pseudomonas stuzeri* and *Lactobacillus fermentum* groups, with 100% similarity. They were named as *Pseudomonas stuzeri* HT2 and *Lactobacillus plantarum* HT2 (Fig. 2).

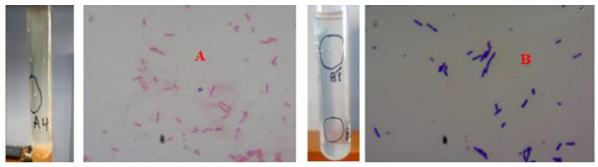

Fig. 1. Results of colony formation in test tubes and Gram staining of *Pseudomonas stuzeri* HT2 **(A)** and *Lactobacillus fermentum* HT2 **(B)**

3.2 Effect of pH

Fig. 3 shows that *Pseudomonas stuzeri* HT2 grew well and oxidised nitrite at pH 6–8.5. The maximum nitrite metabolism rate reached at pH 7 on day 2 with a cell concentration of 4.2 × 10^8 CFU·mL^{-1}.

Lactobacillus fermentum HT2 developed well at pH 5–8 and reached a cell concentration peak at pH 7 on day 2, but the value is much lower (1.1 × 10^8 CFU·mL^{-1}) than that of *Pseudomonas stuzeri* HT2. Thus, pH 7 was selected for further experiments.

Description	Common Name	Max Score	Total Score	Query Cover	E value	Per Ident	Acc. Len	Accession
Pseudomonas stutzeri strain FN9 16S ribosomal RNA gene, partial sequence	Pseudomonas stutzeri	938	938	100%	0.0	100.00%	1481	MT568614.1
Pseudomonas stutzeri Og1 gene for 16S ribosomal RNA, partial sequence	Pseudomonas stutzeri	938	938	100%	0.0	100.00%	848	LC376954.1
Pseudomonas stutzeri strain AAK_MD_30 16S ribosomal RNA gene, partial sequence	Pseudomonas stutzeri	938	938	100%	0.0	100.00%	890	MT180597.1
Pseudomonas stutzeri ATCC 17588 = LMG 11199 16S ribosomal RNA gene, partial seque...	Pseudomonas stutzeri ATCC 175...	938	938	100%	0.0	100.00%	1472	MT027239.1
Pseudomonas stutzeri strain KRP6 16S ribosomal RNA gene, partial sequence	Pseudomonas stutzeri	938	938	100%	0.0	100.00%	1458	MN857682.1

Description	Common Name	Max Score	Total Score	Query Cover	E value	Per Ident	Acc. Len	Accession
Limosilactobacillus fermentum strain AGR1485 chromosome, complete genome	Limosilactobacillus fermentum	1021	5079	100%	0.0	100.00%	2226862	CP047584.1
Lactobacillus fermentum strain MBC0001 16S ribosomal RNA gene, partial sequence	Limosilactobacillus fermentum	1021	1021	100%	0.0	100.00%	1497	MT071599.1
Limosilactobacillus fermentum strain B1.23 chromosome	Limosilactobacillus fermentum	1021	5079	100%	0.0	100.00%	1906587	CP039750.1
Lactobacillus fermentum strain S1 16S ribosomal RNA gene, partial sequence	Limosilactobacillus fermentum	1021	1021	100%	0.0	100.00%	1531	MK226442.1
Limosilactobacillus fermentum strain MTCC 5898 chromosome	Limosilactobacillus fermentum	1021	5054	100%	0.0	100.00%	2096685	CP035904.1

Fig. 2. Results of 16S rDNA sequence comparison of isolated bacteria with NCBI database of *Pseudomonas stuzeri* HT2 **(A)** and *Lactobacillus fermentum* HT2 **(B)**

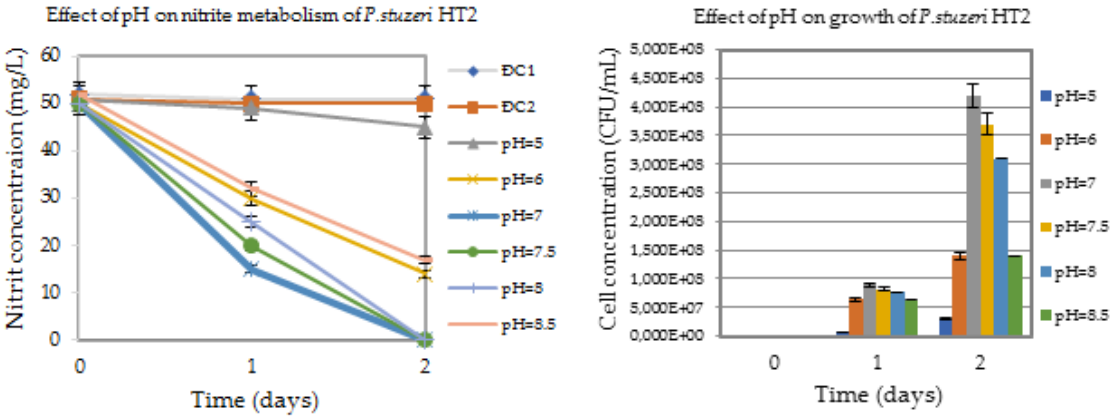

Fig. 3. Effect of pH on growth, development, and nitrite metabolism of *P. stuzeri* HT2

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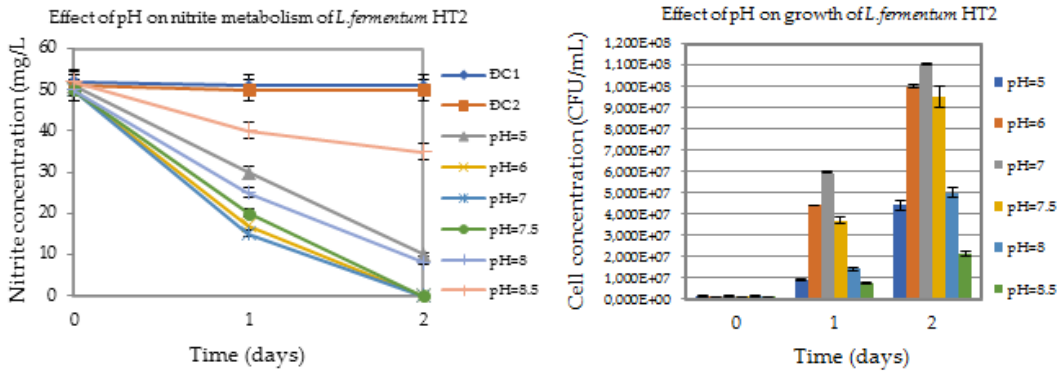

Fig. 4. Effect of pH on growth, development, and nitrite metabolism of *L. fermentum* HT2

3.3 Effect of dissolved oxygen and temperature

The DO concentrations at 0.1, 4.5, and 7.0 mg·L^{-1} were investigated for *P. stuzeri* HT2 and *L. fermentum* HT2 in the pH 7 medium. The results show that both isolated bacteria strains could grow, develop and oxidise nitrite at all tested DO levels. In addition, there was no difference in the nitrite oxidation rate among the tested tubes with various dissolved oxygen levels (Fig.s 5 and 6).

Regarding temperature impacts, the findings reveal that both isolated strains were mesophilic bacteria, exhibiting the highest nitrite oxidation rate at 37 °C. At 30, 45, and 50 °C, they showed some activities but were completely inactive at 5 °C. Therefore, 37 °C was selected for further experiments (Fig.s 7 and 8).

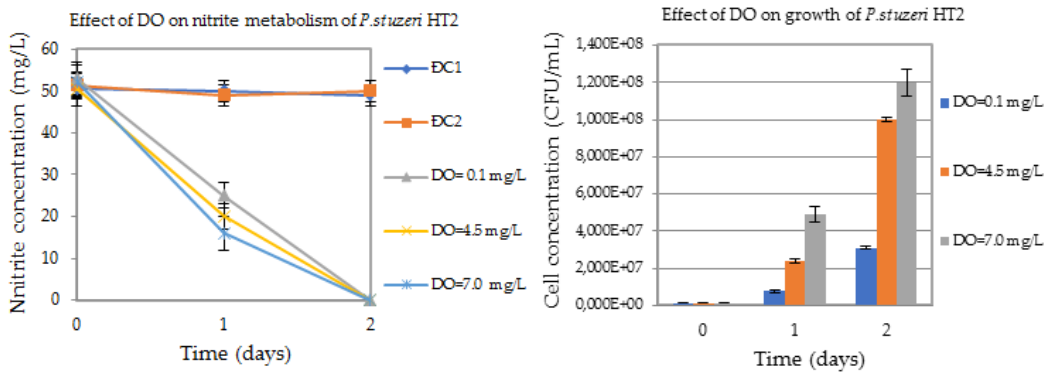

Fig. 5. Effect of DO on growth, development, and nitrite metabolism of *P. stuzeri* HT2

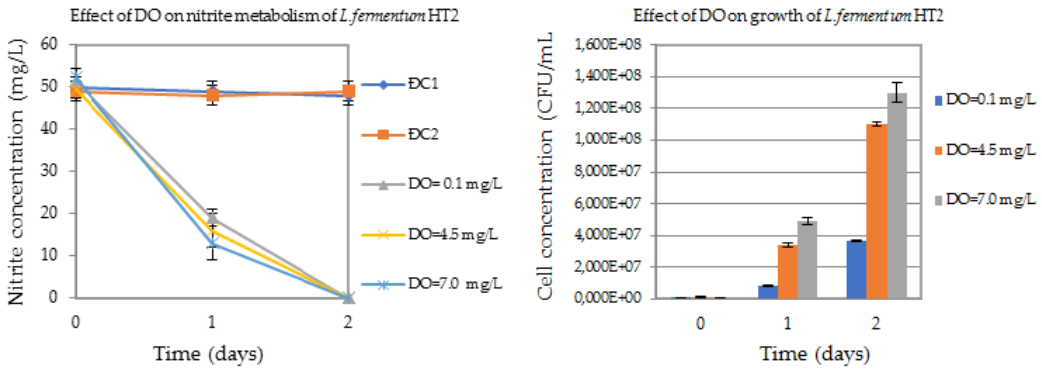

Fig. 6. Effect of DO on growth, development, and nitrite metabolism of *L. fermentum* HT2

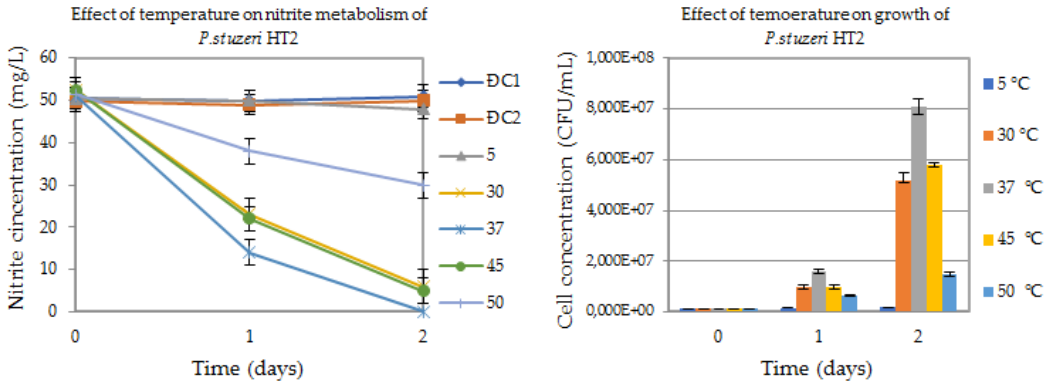

Fig. 7. Effect of temperature on growth, development, and nitrite metabolism *P. stuzeri* HT2

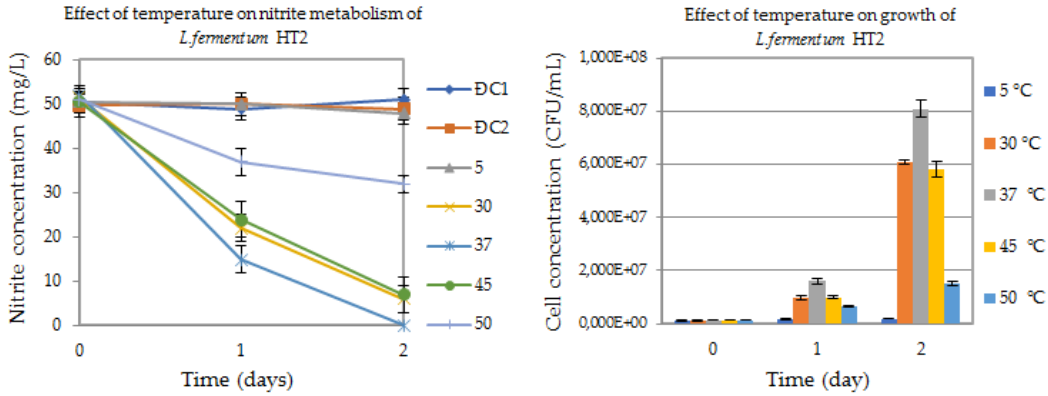

Fig. 8. Effect of temperature on growth, development and nitrite metabolism of *L. fermentum* HT2

3.4 Effect of NaCl concentration

The effect of NaCl concentration on the nitrite metabolism of two isolates was determined in a mineral medium with pH 7 at 37 °C and NaCl concentration at 1, 3, and 5%. The results show that the bacteria worked well at 1 and 3% NaCl concentrations. Meanwhile, the medium with 5% NaCl did not facilitate the tested strains' activity (Fig. 9).

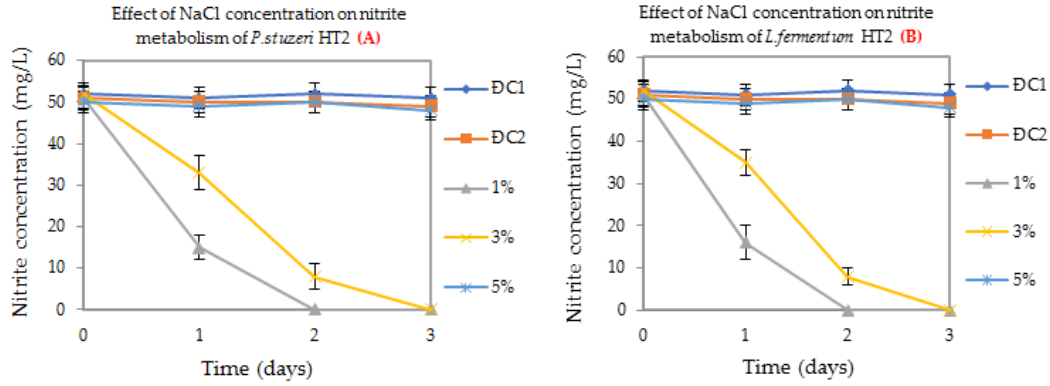

Fig. 9. Effect of NaCl concentration on nitrite metabolism of two bacterial strains *P. stuzeri* HT2 (**A**) and *L. fermentum* HT2 (**B**)

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3.5 Effect of initial nitrite concentration

Initial nitrite concentrations of 100, 500, and 700 mg·L^{-1} were tested under optimal experimental conditions (pH 7 at 37 °C). The results show that both tested bacterial strains could completely metabolise nitrite with concentrations up to 750 mg·L^{-1} after four days of culture. In addition, the nitrite metabolism rate speeded up on the second days for all three tested nitrite concentrations, while the first 24 h of culture observed a slow degradation of nitrite concentration. Interestingly, nitrite metabolism rate was proportional to the initial nitrite concentration (Fig. 10).

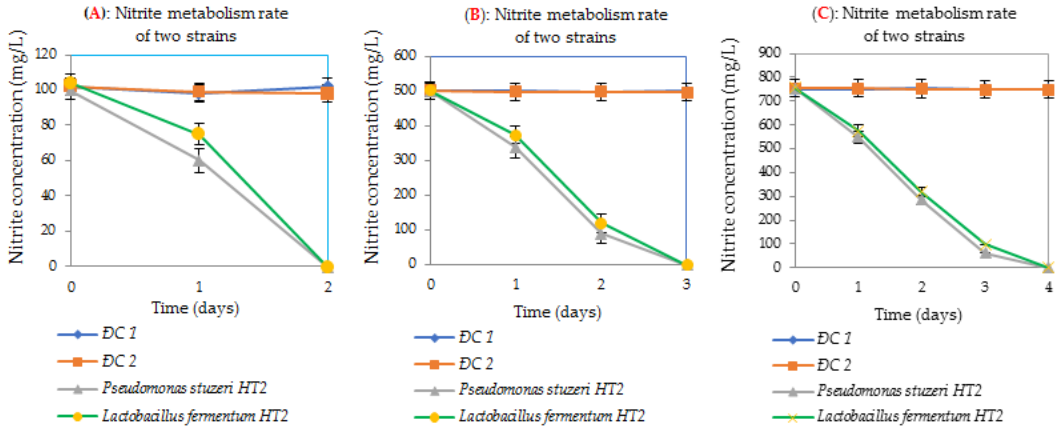

Fig. 10. Nitrite metabolism rate of two bacterial strains with various initial nitrite concentrations: 100 mg·L^{-1} (**A**); 500mg·L^{-1} (**B**); and 750 mg·L^{-1} (**C**)

3.6 Discussions

In this study, two strains of bacteria, *Lactobacillus fermentum* HT2 and *Pseudomonas stuzeri* HT2, isolated from slaughterhouse wastewater and wastewater after biogas treatment in a pig farm, could convert nitrite to nitrate. This finding is in good agreement with other studies published elsewhere [28-33], [34-36]. In other words, they are crucial not only in the natural nitrogen cycle but also in the biological treatment of nitrogen. The results show that they grew and developed well in the prepared mineral medium with insufficient nutrients suitable for autotrophic bacteria. The bacterial density of the strains increased exponentially after only two days of culture, implicating that although the two isolates are heterotrophic bacteria, they have high adaptability to different habitats.

The study shows that the nitrification rate also increased when the tested nitrite concentration increased. This means that the initial nitrite concentration enhanced the oxidation rate of the isolates. Specifically, the bacteria oxidised nitrite substantially at the nitrite concentration of 750 mg·L^{-1}, and the metabolism rate increased with the nitrite concentration. This finding confirms the great potential for applying these strains in biotechnological treatment.

Regarding the effects of dissolved oxygen on the growth and nitrite metabolism of the tested strains, the results show that both bacterial strains could oxidise nitrite under oxygen-deficit conditions (0.1 mg·L^{-1}). In addition, note that the rate of nitrite metabolism is proportional to the oxygen concentration of the culture medium. This issue is confirmed by many strains of *Lactobacillus fermentum* [38] and *Pseudomonas stuzeri* [39] that can grow under anoxic conditions. The ability to grow and metabolise nitrite in an environment with low oxygen concentration demonstrates the potential application of the isolated bacterial

strains in the nitrite treatment of wastewater, especially in waste stabilization ponds with low DO. Similarly, the results of the salt tolerance experiments show that both *Lactobacillus fermentum* HT2 and *Pseudomonas stuzeri* HT2 strains could grow, develop and metabolise nitrite in the medium with a wide range of salinity, i.e. from the freshwater environment to seawater (giving that 3% of NaCl equivalent to the salinity of seawater). This again proves the vital role of these bacterial strains in the natural nitrogen cycle.

In addition, the fact that nitrite oxidation was not observed in any control tests (ĐC1 – medium containing nitrite without the addition of bacteria and ĐC2 – media supplemented with fully autoclaved microbial products) demonstrates that the nitrite metabolism in the test tubes was due to the bacterial strains in the culture medium.

Nowadays, different groups of bacteria have been reported to participate in the nitrite metabolism process [5-19, 28-36]. However, the nitrite-oxidising ability of the bacteria is still limited, which causes Nitrobacter still to be an essential and widespread bacterium in nitrite metabolism not only in nature but also in wastewater treatment. Therefore, the isolation of the two bacterial strains belonging to the Lactobacillus and Pseudomonas groups confirmed the diversity of bacteria that metabolise nitrite in nature. In addition, their conversion capacity and outstanding properties have demonstrated their potential application in the treatment of nitrogen in wastewater.

4 Conclusion

In this study, we successfully isolated two pure strains of bacteria and named them *Lactobacillus fermentum* HT2 and *Pseudomonas stuzeri* HT2. Both strains could metabolise nitrite at up to 750 $mg \cdot L^{-1}$ concentration. In addition, they could adapt to the salted environment with 3% NaCl and oxidised nitrite under conditions with very low DO. The bacteria are prospective for the biological nitrogen removal from wastewater.

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Conflict of interest

The authors have no conflicts of interest regarding the publication of this article.

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