Original Research

Sustainable treatment of nitrate-containing wastewater by an autotrophic hydrogen-oxidizing bacterium

Yi-Zhen Chen a, Li-Juan Zhang a, **, Ling-Yun Ding a, b, Yao-Yu Zhang a, Xi-Song Wang a, Xue-Jiao Qiao a, Bao-Zhu Pan c, Zhi-Wu Wang d, Nan Xu a, Hu-Chun Tao a, * a Key Laboratory for Heavy Metal Pollution Control and Reutilization, School of Environment and Energy, Peking University Shenzhen Graduate School, Shenzhen, 518055, Guangdong, China
b College of Health Science and Environmental Engineering, Shenzhen Technology University, Shenzhen, 518118, Guangdong, China
c Key Laboratory for Heavy Metal Pollution Control and Reutilization, College of Civil and Environmental Engineering, Virginia Polytechnic Institute and State University, Manassas, 20110, Virginia, USA

d State Key Laboratory of Eco-hydraulic in Northwest Arid Region of China, Xi’an University of Technology, Xi’an, 710048, Shaanxi, China

A R T I C L E  I N F O

Article history:
Received 9 November 2021
Received in revised form 19 January 2022
Accepted 20 January 2022

Keywords:
Wastewater
Nitrate
Hydrogen-oxidizing bacteria
Autotrophic assimilation
Aerobic denitrification

A B S T R A C T

Bacteria are key denitrifiers in the reduction of nitrate (NO3-N), which is a contaminant in wastewater treatment plants (WWTPs). They can also produce carbon dioxide (CO2) and nitrous oxide (N2O). In this study, the autotrophic hydrogen-oxidizing bacterium Rhodoblastus sp. TH20 was isolated for sustainable treatment of NO3-N in wastewater. Efficient removal of NO3-N and recovery of biomass nitrogen were achieved. Up to 99% of NO3-N was removed without accumulation of nitrite and N2O, consuming CO2 of 3.25 mol for each mole of NO3-N removed. The overall removal rate of NO3-N reached 1.1 g L−1 h−1 with a biomass content of approximately 0.71 g L−1 within 72 h. TH20 participated in NO3-N assimilation and aerobic denitrification. Results from 15N-labeled-nitrate test indicated that removed NO3-N was assimilated into organic nitrogen, showing an assimilation efficiency of 58%. Seventeen amino acids were detected, accounting for 43% of the biomass. Nitrogen loss through aerobic denitrification was only approximately 42% of total nitrogen. This study suggests that TH20 can be applied in WWTP facilities for water purification and production of valuable biomass to mitigate CO2 and N2O emissions.

© 2022 Published by Elsevier B.V. on behalf of Chinese Society for Environmental Sciences, Harbin Institute of Technology, Chinese Research Academy of Environmental Sciences. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Greenhouse gas (GHG) emissions from wastewater treatment plants (WWTPs) have attracted increasing attention worldwide. Biological processes play a key role in treatment of wastewater containing oxidized contaminants such as nitrate (NO3-N). Heterotrophic denitrification bacteria in these processes release considerable amounts of carbon dioxide (CO2) [1]. In an incomplete denitrification process, denitrification bacteria can also produce nitrous oxide (N2O) and dinitrogen (N2) [2]. The global warming potential of N2O is 273 times that of CO2 on a 100-year timescale [3]. It has been estimated that the level of reactive nitrogen pollution influencing the climate in 2050 will be up to 56% higher than that in 2010 [4]. This increase will boost demand for sustainable techniques to treat NO3-N in WWTPs.

Certain denitrifiers such as Pseudomonas stutzeri TR2 [5], Vibrio sp. Y1-5 [6], Marinobacter strain NNA5 [7], and Pseudomonas mendocina LXY [8] could feasibly reduce N2O emissions. However, these heterotrophic strains also produce CO2 from organic electron donors during NO3-N removal from wastewater. Autotrophic bacteria are promising candidates to mitigate GHG emissions from NO3-N treatment without the need for added organic compounds [9,10]. They are very attractive for use in WWTPs in terms of nutrient recovery via nitrogen assimilation. Recently, hydrogen-oxidizing bacteria (HOB) have been proposed to improve the sustainability of wastewater treatment through assimilation of nitrogen via rapid autotrophic growth on CO2 as a carbon source and hydrogen (H2) as an energy source [11]. HOB can develop niche adaptations to oxic–anoxic environments [12], which can shed light on the feasibility of GHG mitigation in WWTPs where dissolved oxygen (DO) and NO3-N concentrations fluctuate. On one hand, autotrophic HOB obtain electrons from H2, and can quickly

https://doi.org/10.1016/j.ese.2022.100146

2666-4984/© 2022 Published by Elsevier B.V. on behalf of Chinese Society for Environmental Sciences, Harbin Institute of Technology, Chinese Research Academy of Environmental Sciences. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
generate energy and spontaneously capture CO\textsubscript{2} released by heterotrophic bacteria in wastewater or sludge [13]. On the other hand, inorganic nitrogen is simultaneously assimilated into biomass nitrogen, such as cellular proteins consisting of amino acids [14]. NO\textsubscript{3}--N removal by HOB does not release CO\textsubscript{2} or N\textsubscript{2}O, and value-added products such as single-cell proteins and biopolymers are recovered from removed NO\textsubscript{3}--N [15]. To date, most studies have been carried out using mixed HOB cultures for biomass production [16–18], which makes it difficult to explore complex pathways of nitrogen metabolism. Therefore, novel HOB strains and their specific pathways of nitrogen utilization are highly desirable for sustainable treatment of nitrate-containing wastewater.

In this study, a newly isolated HOB strain, *Rhodoblastus* sp. TH20, was evaluated for NO\textsubscript{3}--N removal from wastewater. The pathways of NO\textsubscript{3}--N reduction were investigated in the presence of O\textsubscript{2}. TH20 grew rapidly on CO\textsubscript{2} and efficiently removed NO\textsubscript{3}--N under the optimum conditions. NO\textsubscript{3}--N was consumed as the predominant electron acceptor without inhibition under an aerobic atmosphere. 15\textsuperscript{N}-labeled-nitrate test, 16S rRNA gene sequencing, and genome-wide scanning were conducted to verify the nitrogen metabolism pathways. Autotrophic TH20 is a potential candidate to develop sustainable methods for mitigation of GHG emissions, reduction of nitrogen loss, and reclamation of clear water in WWTPs.

2. Materials and methods

2.1. Strain isolation and cell culture

TH20 (GenBank No. MK968713.1) was isolated from activated sludge in a WWTP in Guangdong, China (Text S1). The isolated strains (TH20 cells with a volume ratio of 5% and an optical density at 600 nm of 1.20) were cultured in 70 mL of mineral medium in a 250-mL airtight serum bottle. The mineral medium (1.0 L) contained KNO\textsubscript{3} (0.58 g), KH\textsubscript{2}PO\textsubscript{4} (0.5 g), NaHCO\textsubscript{3} (0.5 g), MgSO\textsubscript{4} 7H\textsubscript{2}O (0.2 g), CaCl\textsubscript{2} (0.003 g), (NH\textsubscript{4})\textsubscript{2}Fe(SO\textsubscript{4})\textsubscript{2} 6H\textsubscript{2}O (0.02 g), and trace element solution (0.5 mL). The trace element solution (1.0 L, pH 7.0) contained CoCl\textsubscript{2} 6H\textsubscript{2}O (0.119 g), NiCl\textsubscript{2} 6H\textsubscript{2}O (0.118 g), and CuSO\textsubscript{4} 5H\textsubscript{2}O (0.156 g). The mineral medium was sterilized at 121 °C for 20 min, and was cooled to room temperature before use. The (NH\textsubscript{4})\textsubscript{2}Fe(SO\textsubscript{4})\textsubscript{2} 6H\textsubscript{2}O solution was sterilized by filtration through a 0.22-μm membrane filter, and was injected into the mineral medium separately. The bottle was purged with a mixture of H\textsubscript{2}, O\textsubscript{2}, and CO\textsubscript{2} (70:20:10, v/v) gases [19], and then placed in a rotary shaker for agitation.

2.2. Experimental design

Nitrogen removal by TH20 was tested under different operating parameters with NO\textsubscript{3}--N as the nitrogen source. We tested five different pH values (5.0, 6.0, 7.0, 8.0, and 9.0); adjusted using 6 M NaOH and/or 6 M HCl), temperatures (20 °C, 25 °C, 30 °C, 35 °C, and 40 °C), agitation speeds (80, 120, 160, 200, and 240 rpm), and initial NO\textsubscript{3}--N concentrations (0, 20, 50, 80, and 100 mg L\textsuperscript{−1}; adjusted by adding KNO\textsubscript{3} to the mineral medium). When NO\textsubscript{3}--N and NO\textsubscript{2}--N + NH\textsubscript{4}--N were added as nitrogen sources, the fate of nitrogen was determined under the optimum parameters. The concentrations of different nitrogen species (NO\textsubscript{3}--N, NO\textsubscript{2}--N, NH\textsubscript{4}--N, and total nitrogen) were measured. Gas mixtures were tested every 24 h, i.e., at 0, 24, 48, and 72 h, headspace gas (10 mL) was collected to determine H\textsubscript{2}, O\textsubscript{2}, and CO\textsubscript{2} consumption and NO, N\textsubscript{2}O, and N\textsubscript{2} production. Meanwhile, the exhausted headspace gas was replaced with a mixture of H\textsubscript{2}, O\textsubscript{2}, and CO\textsubscript{2} (70:20:10, v/v). All experiments were performed in triplicate unless otherwise specified.

A15\textsuperscript{N}-labeled-nitrate test was carried out to investigate nitrogen transformation during NO\textsubscript{3}--N removal by TH20. The NO\textsubscript{3}--N in the culture medium was replaced with 15NO\textsubscript{3}--N (15% amount-of-substance fraction; Shanghai Research Institute of Chemical Industry, Shanghai, China) at an initial concentration of 80 mg L\textsuperscript{−1}. The test was conducted at pH 7.0 and 25 °C with an agitation speed of 200 rpm. After incubation for 36 h, samples were taken for analyses of 15NO\textsubscript{3}--N, 15NO\textsubscript{2}--N, 15NH\textsubscript{4}--N, 15Norg-N, 15NO\textsubscript{2}, 15N\textsubscript{2}O, and 15N\textsubscript{2}.

16S rRNA gene sequencing and genome-wide scanning were conducted to clarify the specific genes in TH20. Bacterial cells were collected for genome-wide scanning data evaluation and quality control, genome splicing and assembly, and gene prediction and gene annotation. The gene functionalities and pathway maps were derived from the Kyoto Encyclopedia of Genes and Genomes database [20].

2.3. Measurement and analysis

A spectrophotometer (DR6000, Hach, Loveland, CO, USA) was used to determine the NO\textsubscript{3}--N, NO\textsubscript{2}--N, NH\textsubscript{4}--N, and total nitrogen concentrations [21] and to determine the NO\textsubscript{3}--N, NO\textsubscript{2}--N, NH\textsubscript{4}--N, and total nitrogen concentrations [21] and cell optical density at 600 nm. The specific growth rates for TH20 were calculated using the Monod equation: \[ \mu = \frac{S}{K_{\mu} + S}, \] where \( S \) is the concentration of the rate-limiting substrate (mg L\textsuperscript{−1}), \( \mu \) is the specific growth rate (h\textsuperscript{−1}), \( K_{\mu} \) is the maximum specific growth rate (h\textsuperscript{−1}), and \( \mu_{\max} \) is the concentration giving one-half the maximum rate (mg L\textsuperscript{−1}) [22]. The dry cell weight (DCW) was determined by centrifugation of the biomass at 4 °C and 10,000 rpm for 3 min, followed by evaporation at 105 °C for 2 h [23]. Dissolved gases were purged from the culture medium with helium gas at 40 mL min\textsuperscript{−1} for 10 min. Headspace gases (10 mL) were determined using a gas chromatograph (7890B, Agilent, Santa Clara, CA) equipped with a thermal conductivity detector for O\textsubscript{2}, CO\textsubscript{2}, NO, N\textsubscript{2}O, and N\textsubscript{2}, and a flame ionization detector for H\textsubscript{2} [24,25]. Stable 15\textsuperscript{N} isotope ratios were analyzed by chemical conversion and combustion methods using an isotope analyzer (IsoPrime100, Elementar, Langenselbold, Germany). The functional groups in the biomass components were characterized by Fourier transform infrared spectrometry (Spectrum 100, PerkinElmer, Waltham, MA, USA). A CHNS/O elemental analyzer (Vario EL cube, Elementar, Germany) was used to analyze the elemental composition of the biomass. The expression levels of genes related to nitrogen metabolism with different nitrogen sources were investigated by real-time quantitative polymerase chain reaction (qRT-PCR). Genomic RNA was extracted using a Sangon Bacterial RNA Kit (BS11321, Sangon Biotech, Shanghai, China), and cDNA synthesis was performed. According to the gene sequences derived from genome-wide scanning, PCR primers were designed using Primer 5.0 software (PREMIER Biosoft, San Francisco, CA, USA). Amplification was performed with a thermal cycler (TC-XP-G, Bioer Technology, Hangzhou, China). PCR was conducted on a 25-μL sample with initial denaturation at 95 °C for 3 min, followed by 35 cycles of 30 s at 95 °C, 30 s at 57 °C, and 30 s at 72 °C, and then a final extension at 72 °C for 8 min. All quantitative amplifications were performed in triplicate on a StepOne PCR instrument (Thermo Fisher Scientific, Waltham, MA) with a SYBR Green qRT-PCR Kit (B639271, Sangon Biotech, Shanghai, China) and the relevant primers. All trials were performed in parallel (n = 6). The activity of nitrous oxide reductase (N\textsubscript{2}OR) was measured by an enzyme-linked immunosorbent assay following the kit instruction manual (JK142666, Enzyme Linked Biotechnology, Shanghai, China).

3. Results and discussion

3.1. Nitrate removal by autotrophic TH20

TH20 was able to consume NO\textsubscript{3}--N for autotrophic growth under aerobic conditions. The efficacy of TH20 in NO\textsubscript{3}--N removal was
affected by the temperature, pH, agitation speed, and initial NO$_3$-N concentration.

Generally, most microorganisms that can remove NO$_3$-N are sensitive to the culture temperature because it can affect the function of all enzymes involved in the auto-hydrogenotrophic NO$_3$-N removal process [26]. TH20 grew most rapidly at 25 °C (Fig. 1a), reaching a $\mu_{\text{max}}$ of 0.10 h$^{-1}$ in the early logarithmic period (24–36 h). The time needed to achieve rapid cell propagation decreased at higher temperature. At 25–35 °C, TH20 had a relatively short adaptive period and then entered a logarithmic growth period after 12 h. However, TH20 entered a decay period after 36 h at 35 °C possibly because of the formation of a large amount of NO$_2$-N (27.35 ± 0.61 mg L$^{-1}$, Table S1). There was consequently a sharp decrease in the NO$_3$-N concentration from 24 h to 36 h. Cell growth of TH20 was inhibited by accumulation of NO$_2$-N, which suggested that the impact of changes in the temperature on nitrite reductase was more notable than that of NO$_3$-N reductase. Cell growth of TH20 was markedly inhibited at 40 °C. The $\mu_{\text{max}}$ decreased to 0.06 h$^{-1}$ at 12 h under this condition, and then TH20 entered a decay period. From 24 h onwards, the NO$_3$-N concentration increased from 18.63 ± 0.20 mg L$^{-1}$ to its highest concentration (28.04 ± 0.08 mg L$^{-1}$) at 72 h. At 25 °C, the overall NO$_3$-N removal rate was approximately 1.1 mg L$^{-1}$ h$^{-1}$. More than 99% of NO$_3$-N was removed throughout the testing period. Therefore, 25 °C was the optimum temperature for TH20 growth.

Cell growth of TH20 was slightly affected by pH in the tested range (5.0–9.0). The $\mu_{\text{max}}$ changed slightly (Fig. 1b); however, TH20 cells grew better under neutral and weakly alkaline conditions than under stronger alkaline and acidic conditions. The dominant species in solution under neutral and weakly alkaline conditions was HCO$_3$ (Fig. S1), contributing more significantly as carbon source to support the growth of TH20. Similar results have been reported for hydrogenotrophic denitrifying bacteria, which grew faster when HCO$_3$ was used as the sole carbon source [27]. When the pH was lower than 7.0 or higher than 8.0, cell growth of TH20 decreased. The NO$_3$-N removal from aqueous solution was strongly related to the pH value. The lowest NO$_3$-N removal rate (0.90 mg L$^{-1}$ h$^{-1}$) was obtained at pH 5.0. The removal efficiency at this pH value was 83%. Expression of the nirS and cnorB genes in Pseudomonas mandelii also greatly decreased at pH 5.0; thus, removal of NO$_3$-N was inhibited [28]. Therefore, expression of key enzymes in NO$_3$-N removal by TH20 and related functional genes was proposed to also be affected by pH. In order to achieve higher NO$_3$-N removal accompanied by considerable cell growth, the pH value was optimized at 7.0.

The agitation speed affected gas dissolution and transfer into or release from the aqueous phase [29]. Dissolved H$_2$ and DO simultaneously affected the growth of TH20. The solubility of H$_2$ is low (1.6 mg L$^{-1}$ at 20 °C) and the concentration of dissolved H$_2$ in the bulk solution is very dependent on the agitation speed. The best cell growth and NO$_3$-N removal with TH20 were obtained at an agitation speed of 200 rpm (Fig. 1c). When the speed was decreased to 80 rpm, only 55% of NO$_3$-N was removed within 72 h. The $\mu_{\text{max}}$ occurred at 48 h, which was later than under other agitation speeds. With this agitation speed, TH20 did not have enough dissolved H$_2$ as energy to grow. Accumulation of NO$_3$-N occurred with a maximum concentration of 13.81 ± 0.03 mg L$^{-1}$ at 36 h (data not shown). At 200 rpm, the amount of dissolved H$_2$ could meet the requirements for cell growth, which was not inhibited by DO. When the agitation speed was lower than 200 rpm, the $\mu_{\text{max}}$ was elevated by higher contents of dissolved H$_2$. The decreased cell growth of TH20 at 240 rpm may be caused by inhibition with a high concentration of DO. A DO threshold for aerobic NO$_3$-N removal has been proposed and indicated that the conversion rates of NO$_3$-N and DO can be controlled through three routes (Fig. S2). Most aerobic microorganisms consume NO$_3$-N through route II, some through route III, and a few through route I [30]. The NO$_3$-N consumption by TH20 increased from the agitation speed of 80 rpm–200 rpm, and then decreased at 240 rpm, which was likely to follow pathway II.

The effects of the initial NO$_3$-N concentration on TH20 growth and NO$_3$-N removal were investigated (Fig. 1d). The optimum NO$_3$-N concentration for TH20 growth was 80 mg L$^{-1}$, and vigorous growth occurred under this condition. For all concentrations, TH20 was in an adaptation period for the first 12 h, and the growth patterns did not differ much. After entering a logarithmic phase at low NO$_3$-N concentrations that were not sufficient for the growth of TH20, the growth patterns began to show differences. Hence a sufficient supply of nitrogen is crucial to cell propagation. With an initial NO$_3$-N concentration of 80 mg L$^{-1}$, the removal efficiency peaked at approximately 99% and the removal rate was 1.55 mg NO$_3$-N per g DCW per h. This NO$_3$-N removal capability of TH20 was higher than those reported for heterotrophic denitrifiers with and without continuous glucose feed in membrane bioreactors (1.20 and 0.40 mg NO$_3$-N per g DCW per h) [31]. Another study that had similar results to ours showed that the removal rate increased...
with NO₃⁻-N availability in bulk solutions [32]. The first-order kinetics fitted the NO₃⁻-N removal data well in two stages (Fig. S3). Slow NO₃⁻-N removal with a lower rate constant (0.03 h⁻¹) occurred in the adaptive growth period of TH20, and rapid NO₃⁻-N removal with a higher rate constant (0.09 h⁻¹) occurred between 24 and 72 h when the μₘₚ was obtained for TH20 growth. The NO₃⁻-N removal kinetics agreed with the Monod parameters, indicating a high correlation between NO₃⁻-N removal and TH20 growth.

3.2. The fate of nitrate nitrogen used by TH20

Transformation of nitrogen was explored under the optimum growth conditions of TH20 with 80 mg L⁻¹ NO₃⁻-N as the nitrogen source (Fig. 2a). The NO₃⁻-N concentration decreased greatly from 80 mg L⁻¹ to less than 1 mg L⁻¹ after 72 h. This gave a NO₃⁻-N removal efficiency of >99%, which was greater than that by Pseudomonas putida Y-9 (82%) under aerobic conditions [33]. In contrast to other aerobic denitrifiers, such as Pseudomonas stutzeri PCN-1 [34], Paracoccus versutus LYM [35], Pseudomonas sp. ADN-42 [36], and Marinobacter sp. NNA5 [7] that primarily converted NO₃⁻-N into N₂, TH20 assimilated NO₃⁻-N to biomass nitrogen (58%) with accompanying aerobic denitrification to N₂ (42%). This contributed to mitigation of nitrogen loss from the biosphere to atmosphere in nitrate-containing wastewater treatment. The transformation rate of NO₃⁻-N to N₂ was markedly higher in the first 24 h. From 36 h onwards, more NO₃⁻-N was converted to biomass nitrogen. It was noted that approximately 4 mg L⁻¹ NH₄⁺-N was detected during NO₃⁻-N reduction between 12 and 24 h, and this then decreased steadily from 24 to 72 h. As a ubiquitous contaminant in wastewater, NH₄⁺-N was more favorable than NO₃⁻-N for assimilation into biomass nitrogen by TH20 (Fig. 2b). When NO₃⁻-N (40 mg L⁻¹) and NH₄⁺-N (40 mg L⁻¹) were used as the nitrogen sources, the overall efficiency of aerobic denitrification decreased to 22% and more nitrogen was assimilated into biomass nitrogen (78%) than when NO₃⁻-N (80 mg L⁻¹) was used as the nitrogen source. Bacterial cell synthesis competed with aerobic denitrification of NO₃⁻-N to N₂ for electrons and energy in the presence of NH₄⁺-N. Neither NO₃⁻-N nor NO₂⁻ was observed during the transformation of NO₃⁻-N and NH₄⁺-N by TH20 under the optimum conditions. Hence NO₃⁻-N was efficiently assimilated into biomass along with CO₂, which reduced GHG emissions from WWTPs.

The results of ¹⁵N-labeled-nitrate test are shown in Fig. 2c. After 36 h of growth in the ¹⁵NO₃⁻-containing medium, ¹⁵NH₄⁺-N was detected at 1.62 mg L⁻¹ and the ¹⁵N isotope ratio was 11.66%. This confirmed that the dissimilatory nitrate reduction to ammonium (DNRA) pathway was associated with production of organic nitrogen. In addition, the biomass nitrogen content was 29.80 mg L⁻¹, with a ¹⁵N abundance of 13.52%. Therefore, autotrophic assimilation (NO₃⁻-N → NH₄⁺-N → biomass nitrogen) was further involved after DNRA, and the assimilation efficiency of autotrophic TH20 (58%–78%) was notably higher than that of heterotrophic Pseudomonas stutzeri T13 (47%) [37]. These results indicated that TH20 could effectively reduce nitrogen and carbon loss to secure the resources in nitrate-containing wastewater.

3.3. Mechanism of nitrate removal by TH20

Aerobic denitrification, DNRA, and ammonium assimilation (glutamine synthetase, EC 6.3.1.2; glutamate synthase, EC 1.4.113; and glutamate dehydrogenase, EC 1.4.1.13) co-occurred during nitrogen removal by TH20 with 80 mg L⁻¹ NO₃⁻-N as the nitrogen source (Figs. 3a and S4). In addition, the nitrogen fixation pathway occurred with evidence of nifDKH genes detected. Similar pathways of nitrogen metabolism but higher levels of assimilation gene expression were detected for TH20 with 40 mg L⁻¹ NO₃⁻-N and 40 mg L⁻¹ NH₄⁺-N as the nitrogen sources (Fig. S5). This indicated there were flexible metabolism modes for TH20 grown on different nitrogen sources.

Twelve genes involved in nitrogen metabolism by TH20 were quantitatively determined by qRT-PCR under the optimum growth conditions (Fig. 3b). In the denitrification pathway, higher expression of genes nirS and nirK (1.32 and 1.37 × 10⁶ copies per μg RNA) for NO₃⁻-N reduction than of genes narG, narL, and narM (4.21, 5.66, and 8.05 × 10⁵ copies per μg RNA) for NO₃⁻-N reduction demonstrated that NO₃⁻-N reduction was faster than NO₂⁻-N reduction. The enzyme reactivity of N₂OR (encoded by gene nosZ) further explained the negligible amount of N₂O produced in aerobic denitrification. The N₂OR activities at 24–36 h were 2–4 times of those at 48–72 h (Fig. 3c), which showed that conversion of NO₂⁻ to N₂ catalyzed by N₂OR was highly efficient in rapid TH20 growth periods. These results explained the minimal accumulation of NO₂⁻-N and N₂O during NO₃⁻-N removal by TH20 via aerobic denitrification. In the NO₃⁻-N assimilation pathway, the abundance of nirB in TH20 was higher than that of nosA, indicating that transformation
of NO$_3$-N → NO$_2$-N → NH$_4$-N occurred. The lower expression of genes $glnA$, $gltB$, and $gltD$ suggested that the formed NH$_4$-N was partly converted into amino acids.

3.4. Amino acid production by TH20

Changes in the amino acid profile were used to verify the conversion of NO$_3$-N into amino acids (Fig. 4). The DCW of TH20 was 0.71 g L$^{-1}$, and 43% of this was composed of seventeen amino acids, including eight essential amino acids. Glutamic acid, aspartic acid, alanine, and leucine were the four most abundant amino acids, with contents higher than 3 g per 100 g DCW. Glutamic acid and aspartic acid are raw materials for the synthesis of nucleotides [38,39], and the high contents of these amino acids in TH20 will facilitate the formation of nucleotides. Leucine is the most effective branched-chain amino acid, and is often involved in protein synthesis and energy metabolism [40,41]. The Fourier transform infrared results (Fig. S6) further demonstrated that NO$_3$-N was transformed into organic nitrogen and stored intracellularly. The elemental composition (Table S2) gave a molecular formula for TH20 cells of C$_{5.60}$H$_{12.69}$O$_{3.52}$N. The nitrogen content of TH20 cells (9.32%) was close to that of the two most abundant amino acids, and higher than that of the autotrophic HOB _Paracoccus versutus_ D6 (8.10%) [23]. Because TH20 primarily assimilated NO$_3$-N into biomass nitrogen via autotrophic assimilation, CO$_2$ was simultaneously fixed into TH20 cells. The content of organic carbon was 44.74%, which was equal to the median content of seventeen amino acids. Therefore, rapid propagation of TH20 cells was evidenced by the changing profile of amino acids. Previous studies have demonstrated that HOB with NO$_3$-N assimilation capability can be applied as biofertilizers [38]; therefore, TH20 could be used as a biofertilizer after NO$_3$-N recovery.

3.5. Carbon sequestration by TH20

TH20 could grow autotrophically on CO$_2$ and efficiently sequester inorganic carbon into the cell biomass. No organic carbon was required, and no CO$_2$ was released. The molar ratios of CO$_2$ uptake against H$_2$ uptake ranged from 0.18 to 0.26 (Fig. 5). These values were consistent with the metabolic parameters for the growth of typical HOB [19]. In the logarithmic growth period, the highest CO$_2$/H$_2$ value was obtained for autotrophic TH20 cells, and the ratio agreed with the stoichiometry of carbon and energy utilization in Table 1. For each mole of NO$_3$-N removed, 3.25 mol of CO$_2$ was consumed. The CO$_2$ fixed by TH20 was approximately 3 mol more than that fixed by mixed autotrophic strains in activated sludge and biofilms [42,43]. By contrast, oxidation of CH$_3$OH and CH$_3$CH$_2$OH as organic carbon sources during removal of 1 mol

---

**Fig. 3.** (a) Nitrogen metabolism pathways with gene abundance (copies per µg RNA), (b) quantification of respective functional genes, and (c) enzyme activity of nitrous oxide reductase (N$_2$OR) in TH20 under the optimum conditions.

**Fig. 4.** Amino acid, nitrogen, and carbon contents of the bacterial biomass produced by TH20 under the optimum conditions.

**Fig. 5.** Consumption of H$_2$, O$_2$, and CO$_2$ gas mixture by TH20 under the optimum conditions.
of NO$_3$-N generated 0.76 mol of CO$_2$ in activated sludge processes and 0.68 mol of CO$_2$ in biofilm processes [22,44]. Net consumption of approximately 4 mol of CO$_2$ was achieved using autotrophic TH20 instead of heterotrophic denitrifying bacteria. In addition, TH20 grew faster for CO$_2$ sequestration and NO$_3$-N assimilation into biomass under aerobic conditions than under anaerobic conditions (Fig. S7). This was possibly because of the large quantities of energy released from the hydrogen oxidation process [45]. In practical application, gaseous substrates of H$_2$ and O$_2$ (together with CO$_2$) need to be produced by green techniques such as microbial electrolysis cells with smaller power supplies [46]. Explosive H$_2$ and O$_2$ should always be treated with the utmost caution. With the implementation of green and safe processes, it will be possible to exploit TH20 for mitigation of GHG emissions in sustainable WWTPs.

4. Conclusions

The newly isolated HOB strain, *Rhodoblastus* sp. TH20, effectively removed 99% of NO$_3$-N with an overall removal rate of approximately 1.1 mg L$^{-1}$ h$^{-1}$. Autotrophic assimilation, aerobic denitrification, and DNRA pathways co-occurred with TH20. The NO$_3$-N was primarily removed by autotrophic assimilation with synthesis of valuable amino acids and consumption of CO$_2$. No accumulation of NO$_2$-N and N$_2$O occurred in the aerobic denitrification pathway. TH20 exhibited both efficient NO$_3$-N removal and rapid recovery of biomass nitrogen. The excellent potential of autotrophic TH20 makes it a promising candidate to mitigate CO$_2$ and N$_2$O emissions in sustainable treatment of nitrate-containing wastewater.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was funded by the Shenzhen Fundamental Research Programs (JCY20180503182122539, JCY20180503182130795, and GXWD20201231165807007-20200810165349001) and the National Natural Science Foundation of China (51939009). We appreciate the suggestions and help of Dr. Wei-Min Wu from Stanford University during manuscript preparation.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ese.2022.100146.

Table 1

| Strain          | Carbon source | Reaction | CO$_2$ emission (mol per mol nitrate) | Reference |
|-----------------|---------------|----------|---------------------------------------|-----------|
| *Rhodoblastus* sp. TH20 | CO$_2$ | NO$_3^-$ + 10.64 H$_2$ + H$^+$ + 3.25 CO$_2$ − 0.58 C$_6$H$_{12}$O$_6$15$_2$N$_2$ + 0.21 N$_2$ + 7.46 H$_2$O | −3.25 | This study |
| Activated sludge | CO$_2$ | NO$_3^-$ + 2.82 H$_2$ + H$^+$ + 0.14 CO$_2$ − 0.028 C$_6$H$_{12}$O$_6$N + 0.49 N$_2$ + 3.22 H$_2$O | −0.14 | [42] |
| Biofilm         | CO$_2$ | NO$_3^-$ + 3.35 H$_2$ + H$^+$ + 0.37 CO$_2$ − 0.074 C$_6$H$_{12}$O$_6$N + 0.46 N$_2$ + 3.59 H$_2$O | −0.37 | [43] |
| Activated sludge | CH$_3$OH | NO$_3^-$ + H$^+$ + 1.08 CH$_3$OH −0.065 C$_6$H$_{12}$O$_6$N + 0.47 N$_2$ + 0.76 CO$_2$ + 2.44 H$_2$O | 0.76 | [44] |
| Biofilm         | CH$_3$CH$_2$OH | NO$_3^-$ + H$^+$ + 0.67 CH$_3$CH$_2$OH −0.13 C$_6$H$_{12}$O$_6$N + 0.43 N$_2$ + 0.68 CO$_2$ + 2.04 H$_2$O | 0.68 | [23] |

References

[1] H.Y. Zheng, Y. Liu, X.Y. Gao, G.M. Ai, LL. Miao, Z.P. Liu, Characterization of a marine origin aerobic nitrifying-denitrifying bacterium, J. Biosci. Bioeng. 114 (1) (2012) 33–37.
[2] G. Wesgothero, T. Hein, Efficiency and detrimental side effects of denitrifying bioreactors for nitrite reduction in drainage water, Environ. Sci. Pollut. Res. 22 (17) (2015) 13534–13545.
[3] P. Forster, T. Storelvmo, K. Armer, W. Collins, J.L. Dufrasne, B. Frame, D.J. Lunt, T. Mauritzen, J.S. Palmer, M. Watanabe, M. Wild, H. Zhang, The Earth’s energy budget, climate feedbacks, and climate sensitivity, in: Climate Change 2021: The Physical Science Basis. Contribution of Working Group I to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, 2021.
[4] B.L. Bodirsky, A. Popp, H. Lotze-Campen, J.P. Dietrich, S. Rolinski, I. Weindl, C. Schmitz, C. Müller, M. Bonisch, P. Humpenöder, Reactive nitrogen requirements to feed the world in 2050 and potential to mitigate nitrogen pollution, Nat. Commun. 5 (2014), 3856.
[5] M. Miyahara, S.-W. Kim, S. Fusihinobu, K. Takaki, T. Yamada, A. Watanabe, K. Myauchi, G. Endo, T. Wakagi, H. Shoun, Potential of aerobic denitrification by Pseudomonas stutzeri TR2 to reduce nitrous oxide emissions from waste-water treatment plants, Appl. Environ. Microbiol. 76 (14) (2010) 4619–4625.
[6] Y.T. Li, Y.R. Wang, L. Fu, Y.Z. Gao, H.X. Zhou, W.Z. Zhou, Aerobic-heterotrophic nitrogen removal through nitrite reduction and ammonium assimilation by marine bacterium Vibrio sp. Y1-5, Biosci. Technol. 230 (2017) 103–111.
[7] Y. Liu, G.M. Ai, LL. Miao, Z.P. Liu, Marinobacter strain NNA5, a newly isolated and highly efficient aerobic denitrifier with zero N$_2$O emission, Biosci. Technol. 206 (2016) 9–15.
[8] Y. Li, J. Ling, P. Chen, J. Chen, R. Dai, J. Liao, J. Yu, Y. Xu, Pseudomonas mendocina LXY: a novel aerobic bacterium with advantage of removing nitrate high effectively by assimilation and dissimilation simultaneously, Front. Environ. Sci. Eng. 15 (4) (2021), 57.
[9] P.F. Capua, F. Pirozzi, P.N.L. Lens, G. Esposito, Electron donors for autotrophic denitrification, Chem. Eng. J. 362 (2019) 922–937.
[10] Y.P. Zhang, G.B. Douglas, A.H. Kaksonen, LL. Cui, Z.F. Ye, Microbial reduction of nitrogen in the presence of zero-valent iron, Sci. Total Environ. 646 (2019) 1195–1203.
[11] S. Matassa, N. Boon, W. Verstraete, Resource recovery from used water: the manufacturing abilities of hydrogen-oxidizing bacteria, Water Res. 68 (2015) 467–478.
[12] X. Hu, P. Vandamme, N. Boon, Co-cultivation enhanced microbial protein production based on autotrophic nitrogen-fixing hydrogen-oxidizing bacteria, Chem. Eng. J. 429 (2022) 132535.
[13] L.J. Zhang, Y. Xie, L.Y. Ding, X.J. Qiao, H.C. Tao, Highly efficient ammonium removal through nitrogen assimilation by a hydrogen-oxidizing bacterium, *Ideonella* sp. TH17, Environ. Res. 191 (2020), 110059.
[14] P. Li, W. Xing, J. Luo, L.T. Tang, Y.J. Wang, J. Lin, Hydrogenotrophic denitrification for tertiary nitrogen removal from municipal wastewater using membrane diffusion packed-bed bioreactor, Biosci. Technol. 144 (2013) 452–459.
[15] U. Javouzel, M. O'Donhue, L. Hamelin, Waste-to-nutrition: a review of current and emerging conversion pathways, Biotechnol. Adv. 53 (2021) 107857.
[16] S. Matassa, W. Verstraete, I. Pikaar, N. Boon, Autotrophic nitrogen assimilation and carbon capture for microbial protein production by a novel enrichment of hydrogen-oxidizing bacteria, Water Res. 101 (2016) 137–146.
[17] X. Hu, F.M. Kerckhofs, J. Ghosequere, K. Bernaerts, P. Boeckx, P. Claewert, N. Boon, Microbial protein out of thin air: fixation of nitrogen gas by an autotrophic hydrogen-oxidizing bacterial enrichment, Environ. Sci. Technol. 54 (6) (2020) 3609–3617.
[18] W. Zhang, F. Zhang, Y. Niu, Y.X. Li, Y.J. Yang, Y.N. Bai, K. Dai, R.J. Zeng, Power to hydrogen-oxidizing bacteria: effect of current density on bacterial activity and community spectra, J. Clean. Prod. 263 (2020b) 121596.
[19] T.G. Volova, E.G. Kiselev, E.I. Shishatskaya, N.O. Zhila, A.N. Boyandin, D.A. Sryvachova, D.N. Vinogradova, G.S. Kalacheva, A.D. Vasilev, J.V. Peterson, Cell growth and accumulation of polyhydroxyalkanoates from CO$_2$ and H$_2$: a hydrogen-oxidizing bacterium, *Cupriavidus* *atrospratus* B-10646, Biosci. Technol. 146 (2013) 215–222.
