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Denitrification-Potential Evaluation and Nitrate-Removal-Pathway Analysis of Aerobic Denitrifier Strain *Marinobacter hydrocarbonoclasticus* RAD-2

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Received: 15 August 2018; Accepted: 20 September 2018; Published: 21 September 2018

Abstract: An aerobic denitrifier was isolated from a long-term poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate) PHBV-supported denitrification reactor that operated under alternate aerobic/anoxic conditions. The strain was identified as *Marinobacter hydrocarbonoclasticus* RAD-2 based on 16S rRNA-sequence phylogenetic analysis. Morphology was observed by scanning electron microscopy (SEM), and phylogenetic characteristics were analyzed with the API 20NE test. Strain RAD-2 showed efficient aerobic denitrification ability when using NO_3^--N or NO_2^--N as its only nitrogen source, while heterotrophic nitrification was not detected. The average NO_3^--N and NO_2^--N removal rates were 6.47 mg/(L·h)and 6.32 mg/(L·h), respectively. Single-factor experiments indicated that a 5:10 C/N ratio, 25–40 °C temperature, and 100–150 rpm rotation speed were the optimal conditions for aerobic denitrification. Furthermore, the denitrifying gene *napA* had the highest expression on a transcriptional level, followed by the denitrifying genes *nirS* and *nosZ*. The *norB* gene was found to have significantly low expression during the experiment. Overall, great aerobic denitrification ability makes the RAD-2 strain a potential alternative in enhancing nitrate management for marine recirculating aquaculture system (RAS) practices.

Keywords: aerobic denitrification; *Marinobacter hydrocarbonoclasticus* RAD-2; nitrogen removal; denitrifying gene expression; wastewater treatment

1. Introduction

Recirculating aquaculture systems (RAS) are a potential alternative to traditional aquaculture systems due to their intensive production and environmental sustainability [1]. In practice, RAS mainly use biological filters to oxidize ammonium to nitrate through nitrification, with nitrite as the intermediate product since ammonium and nitrite have direct toxicity to most fish species [2]. Nitrate concentration accumulates and reaches high concentrations during intensive fish farming. Therefore, nitrate management is very important due to its explicit long-term stress effect on cultured species [3], as well as its contribution to environmental eutrophication [4,5]. In various nitrate-removal

methods, biological heterotrophic denitrification was proved to be an efficient approach in wastewater treatment [6]. However, the heterotrophic denitrification process depends highly on sufficient organic substances as electron donors, which inhibit its application under the circumstances of a low C/N ratio, such as groundwater or RAS effluent treatment [4,7]. Therefore, an interesting alternative that uses biodegradable polymers as simultaneous biofilm carriers and carbon sources was proposed and demonstrated as feasible for nitrate removal in many solid-phase denitrification reactors [6–12].

Denitrification based on biodegradable polymers is usually operated under anoxic conditions due to the fact that conventional denitrification processes relied on the activities of four fundamental enzymes, that is, respiratory nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase, to sequentially transform nitrate into N_2 [13]. Meanwhile, the first step that transfers nitrate to nitrite, encoded by the *Nar* gene, was found mostly sensitive to the presence of oxygen [14]. However, this anoxic solid-phase denitrification process also has byproducts due to potential electron-donor competition with other substances in practice. In our previous study, sulfate reduction to toxic sulfide was detected in marine-wastewater treatment, as sulfates are the next best terminal electron acceptor when nitrate is consumed [10]. In addition, high levels of sulfide and salinity might support dissimilatory nitrate reduction to ammonium (DNRA) over denitrification [15], which were also widely detected in other anoxic biodegradable-polymer denitrification systems [10,12,16,17].

To overcome these problems, the solution of applying oxygen to cut off the route of electron transport through DNRA and sulfate reduction under alternant aerobic/anoxic conditions was demonstrated as feasible in our previous study [18]. However, the introduction of oxygen as selective pressure could lead to a more complicated microbial ecology structure [19] due to existing anoxic microzones developed by the gradual degradation of polymer carriers. In addition, many aerobic denitrifiers were reported to have the capacity for nitrate removal under aerobic conditions [20,21]. In the aerobic denitrification process, another electron-transfer pathway was found to be insensitive to oxygen, which relies on the expression of the *napA* gene (encoding periplasmic nitrate reductase) to make these groups respire nitrate and oxygen simultaneously [22,23]. Until now, many aerobic denitrification bacterial species have been reported, including *Thiosphaera pantotropha* [24], *Marinobacter* NNA5 and F6 [20,25], *Zobellella taiwanensis* DN-7 [26], and *Paracoccus versutus* LYM [27].

However, the above-mentioned solution might cause more complicated ecological-niche competition. Hence an opium microbial community is crucial to denitrification potential. Therefore, to enhance nitrate-removal performance in such a solid-phase denitrification system, one potential alternative could be optimizing the microbial community through bioaugmentation. For example, adding the *Diaphorobacter polyhydroxybutyrativorans* strain SL-205 to a solid-phase denitrification reactor could increase nitrate-removal efficiency [28]. The SL-205 strain was isolated from an anoxic poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate) PHBV-supported denitrification reactor [29]. However, few studies have been conducted on isolating strains from an alternant aerobic/anoxic biodegradable-polymer denitrification reactor.

In this study, a strain, *Marinobacter hydrocarbonoclasticus* RAD-2, was isolated from a long-term PHBV-supported denitrification reactor that operated under alternate aerobic/anoxic conditions for marine RAS-effluent treatment. PCR (polymerase chain reaction) amplification of the 16S rRNA gene was performed to identify the isolated strain. In addition, evaluation of its denitrification-potential performance was carried out. Moreover, key denitrifying gene (*napA*, *nirS*, *norB*, and *nosZ*) expression was investigated to illuminate the mechanism of nitrate-removal pathways in the aerobic denitrification process. Overall, our results might provide new microbial resources and potential alternatives for enhancing nitrate-removal performance in marine RAS practices.

2. Materials and Methods

2.1. Culture Media

The denitrification medium (DM) was prepared to investigate the aerobic denitrification ability of strain RAD-2 by dissolving 2.0 g sodium acetate, 2.0 g KNO_3 (or $NaNO_2$), 0.2 g of $MgSO_4 \cdot 7H_2O$,

1.0 g of K_2HPO_4, and 10 mL of a trace-element solution in 1 L of distilled water. The heterotrophic nitrification medium (HNM) was prepared by dissolving 2.0 g sodium acetate, 0.3 g of NH_4Cl, 0.2 g of $MgSO_4{\cdot}7H_2O$, 6.7 g of Na_2HPO_4, 1.0 g of KH_2PO_4, and 10 mL of a trace-element solution in 1 L of distilled water. The composition of the trace-element solution was 50.0 g of EDTA, 2.2 g of $ZnSO_4$, 5.5 g of $CaCl_2$, 5.06 g of $MnCl_2{\cdot}4H_2O$, 5.0 g of $FeSO_4{\cdot}7H_2O$, 1.1 g of $(NH_4)_6Mo_7O_2{\cdot}4H_2O$, 1.57 g of $CuSO_4{\cdot}5H_2O$, and 1.61 g of $CoCl_2{\cdot}6H_2O$ in 1 L of distilled water. The Luria-Bertani (LB) medium was prepared by dissolving 5.0 g yeast extract, 10.0 g peptone, and 25.0 g NaCl in 1 L of distilled water and 1.5% (*w*/*v*) agar. The initial pH of all media was set to 7.2, and all media were autoclaved for 20 min at 121 °C.

2.2. Bacteria Isolation, Screening, and Identification

Strain RAD-2 was isolated from the biofilms of a long-term aerobic/anoxic denitrifying reactor using PHBV as simultaneous carbon source and carrier. The reactor setup and operation conditions were according to our previous study [18]. The reactor was placed in a dark artificial-climate room to retain the temperature at 26 ± 2 °C. The influent NO_3^--N concentration was set at 70 mg/L and HRT (hydraulic retention time) was 4 h. In detail, 20 g of matured PHBV samples and 10 mL solution samples were aseptically transferred to a flask with 100 mL sterile water and 10 small glass balls. To suspend the biofilms attached to the PHBV granules, the flask was shaken on a rotary shaker at 200 rpm for 30 min. The homogenized suspensions were serially diluted and plated using a DM, and then incubated at 28 °C for 72 h. A single colony with a white circle was purified by streaking onto an LB medium plate, which was then incubated for three days at 28 °C. Several colonies were obtained after strict investigation of their purity. Among the isolates, a colony that was white, irregular circle-shaped with opaque, wet, and smooth surfaces, 1–2 mm in diameter was distinguished as RAD-2. The purified isolate was stored in a 30% glycerol solution at −80 °C.

The genomic DNA of the RAD-2 strain was isolated using a DNA extraction kit (TaKaRa Biotechnology Co. Ltd, Beijing, China). The 16S rRNA gene was PCR-amplified using bacterial universal primers F27 (5′–AGAGTTTGATCMTGGCTCAG–3′) and R1492 (5′–TTGGYTCCTTGT TACGACT–3′), under the following conditions: 2 min at 95 °C, 25 cycles of 20 s at 95 °C, 20 s at 55 °C, 30 s at 72 °C, and a final step of 10 min at 72 °C. PCR products were detected on 1% agarose gel electrophoresis and ethidium bromide staining. The amplified products were purified and sequenced by the Zhejiang Institute of Microbiology (Hangzhou, Zhejiang, China). The sequence was submitted to the NCBI database (accession numbers MH725589) and compared with other available 16S rRNA gene sequences in Genbank by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis software (MEGA) version X by the neighbor-joining method with 1000 bootstrap replicates.

2.3. Nitrogen-Removal Performance Evaluation

To evaluate aerobic denitrification capacity, a sole nitrogen source of NO_3^--N (around 300 mg/L) or NO_2^--N (around 300 mg/L) was tested in DM containing KNO_3 or $NaNO_2$, respectively. Afterward, a 3 mL seed suspension was inoculated in 250 mL Erlenmeyer flasks and cultured for 48 h with aeration at 27 °C and 150 rpm. To evaluate the capacity for heterotrophic nitrification, a similar operation was carried out that only replaced the HNM with a sole nitrogen source of NH_4Cl (around TAN 90 mg/L). Cell-growth and inorganic-nitrogen changes were measured every 4 h. The nitrogen-removal rate was calculated as below:

$$R_N = (C_I - C_F) \times V \times 4/1000/T$$

where R_N = nitrogen removal rate, mg/(L·h); C_I = initial NO_3^--N or NO_2^--N concentration, mg/L; C_F = final NO_3^--N or NO_2^--N concentration, mg/L; V = volume, mL; T = incubated time, h.

Single-factor experiments were also carried out to evaluate the effect of various conditions on the aerobic denitrification performance of strain RAD-2. The operation conditions for DM were as

follows: NO_3^--N concentration of around 300 mg/L, C/N ratio 10, NaCl 25%, temperature 25 °C, rotation 150 rpm, and 1.2% inoculation (*v*/*v*). For temperature experiments, the temperature was set to 5 °C, 10 °C, 15 °C, 25 °C, and 40 °C. For C/N ratio experiments, the C/N ratios were set to 2, 5, 10, 15, and 20. For dissolved oxygen (DO) experiments, the rotations were set to 0, 50, 100, 150, and 200 rpm. Cell growth and indexes (nitrate, nitrite, DOC, and pH) were measured during the experimental period. All tests were conducted in triplicate and none-seeded samples were used as blank control.

2.4. RT-qPCR Analysis

To quantitatively analyze the potential aerobic denitrification pathways of strain RAD-2, real-time PCR was conducted to amplify the denitrifying genes *napA*, *nirS*, *norB*, *nosZ*, and 16S rRNA (housekeeping gene) with RNA samples in 48 h experiments. Total RNA extraction and cDNA synthesis were performed by using an RNAprep Bacteria Kit and FastQuant RT Kit (Tian Gen Biotech Co. Ltd, Beijing, China), respectively. Primers are listed in Table S1. PCR amplification was performed with the following protocol: an initial denaturation step of 10 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C (*napA*, *nirS*, and *nosZ*) or 56 °C (16S V3 region and *norB*) for 30 s, and a final extension at 72 °C for 30 s [30]. All quantitative amplifications were conducted in triplicate using the SYBR Green Real-Time PCR Kit (Novland, Shanghai, China) and respective primers on an Mx3000P qPCR System (Agilent Technologies Co. Ltd., Beijing, China) [19].

2.5. Analytical Methods

The solution samples were collected and filtered through a 0.45 μm filter membrane before water-quality analysis. TAN, NO_2^--N, and NO_3^--N concentrations were analyzed according to standard methods [31]. Cell growth (OD_{600}) was measured by using a spectrophotometer at 600 nm. DOC was measured using a TOC analyzer (Multi N/C 2100, Analytik Jena, Jena, Germany). DO was measured using a DO meter (SG9-FK2, Mettler Toledo, Zurich, Switzerland). Morphological analysis was performed by scanning electron microscopy (SEM) (SU8010; HITACHI, Tokyo, Japan). Fresh colonies grown on LB agar for 2 days were fixed in 1% glutaraldehyde (prepared in cacodylate buffer, pH 7.4) at 4 °C overnight, and then completely dehydrated in ethanol. Cells were coated with gold–palladium and observed with a HITACHI 8010 scanning electron microscope (HITACHI, Tokyo, Japan). Physiological and biochemical characteristics were tested using API 20NE kits (BioMérieux Shanghai Co. Limited, Shanghai, China). API 20NE test strips was checked after incubation for 24 h [29].

3. Results

3.1. Characteristics and Identification

In this study, more than six pure isolates were obtained from solid DM and tested for aerobic denitrification performance by monitoring changes in nitrite and nitrate concentration in the liquid DM. A particular isolate, namely, RAD-2, exhibited the highest efficiency in nitrate and nitrite removal and was subject to further investigation. Strain RAD-2 was slightly halophilic and able to grow under aerobic conditions. The colonies of RAD-2 were yellow, small, circular in shape, semitransparent, slabby, and presented a wet surface on the LB medium. The cells were Gram-negative, bacilliform, with a size of 0.3–0.4 μm in diameter and 1.0–2.0 μm in length (Figure S1).

According to API 20 NE tests (Table 1), strain RAD-2 was positive for oxidase, and nitrate was reduced, but it was negative for arginine dihydrolase, urease, β-glucosidase, protease, and β-galactosidase. It could not perform assimilation of arabinose, mannose, mannitol, N-acetyl-glucosamine, maltose, capric acid, adipic acid, malic acid, citric acid, and phenylacetic acid.

Analysis of 16S rRNA gene sequencing showed that strain RAD-2 belongs to the species *Marinobacter hydrocarbonoclasticus*, having 98% similarity with *Marinobacter hydrocarbonoclasticus* strain

ATCC 49840 and *Marinobacter hydrocarbonoclasticus* strain VT8. Phylogenetic analyses of the 16S rRNA gene sequencing showed that strain RAD-2 formed a distinct clade with strain ATCC 49840 and strain VT8, and this clade clustered with the nearest clade containing *Marinobacter* sp. NN5, *Marinobacter* sp. U1369-101122-SW163, and *Marinobacter hydrocarbonoclasticus* strain NY-4 (Figure 1). The phylogenetic position of this strain indicated that it presented a subspecies of the species *Marinobacter hydrocarbonoclasticus*.

Table 1. Characteristics of strain RAD-2 determined by API 20 NE tests.

API 20 NE Results	Strain RAD-2
Oxidase test	+
Nitrate reduction	+
Arginine dihydrolase	-
Urease	-
β-glucosidase	-
Protease	-
β-galactosidase	-
Assimilation of Glucose	+
Arabinose	-
Mannose	-
Mannitol	-
N-acetyl-glucosamine	-
Maltose	-
Gluconate	+
Capric acid	-
Adipic acid	-
Malic acid	-
Citric acid	-
Phenylacetic acid	-

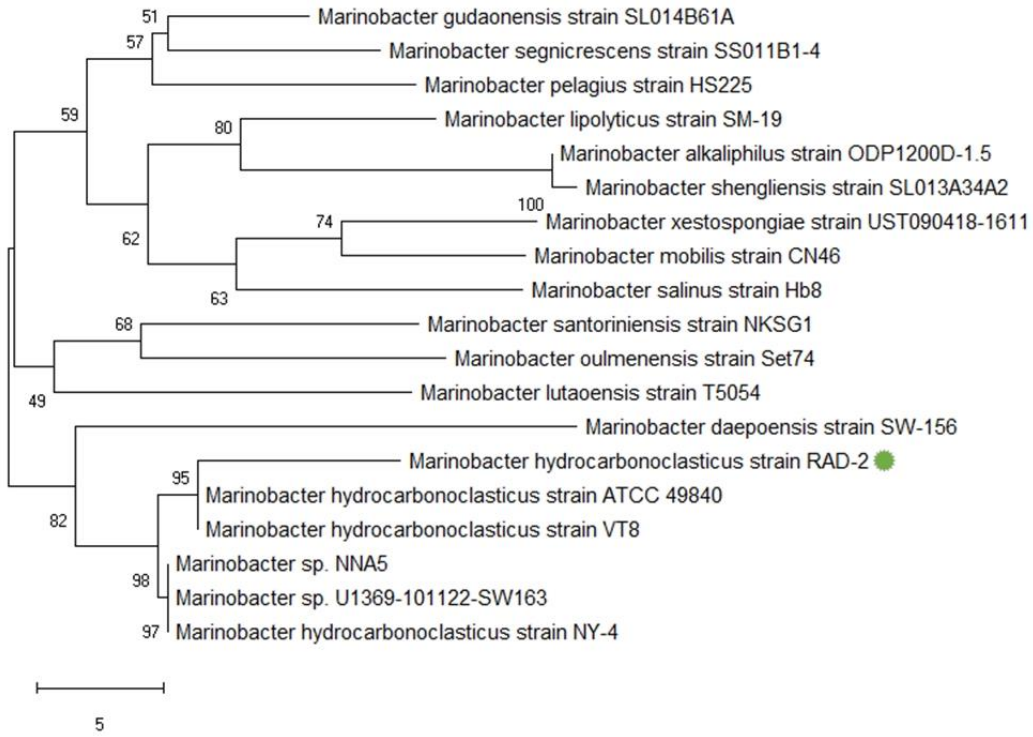

Figure 1. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strain RAD-2 and closely related strains. Bootstrap values based on 1000 replicates are shown at branch nodes.

3.2. Nitrogen Removal Performance Evaluation

3.2.1. Aerobic Nitrogen-Removal Ability of Strain RAD-2

The aerobic denitrification ability of strain RAD-2 under varied nitrogen sources is shown in Figure 2. After 48 h of incubation, NO_3^--N concentration decreased from the initial 310.94 mg/L to the final 5.17 mg/L, which indicated 98.34% removal efficiency (Panel A). The obvious lag phase was observed between 0 and 24 h, while the logarithmic growth phase was observed between 24 and 36 h. Nitrite accumulation occurred between 20 and 36 h, while peak concentration of 5.05 mg/L was observed at 32 h. In addition, a slight ammonium concentration of 2.96 mg/L was also found in the final concentration. The biomass growth of OD_{600} reached 1.34. Additionally, when nitrite was used as the sole nitrogen source (Panel B), NO_2^--N concentration also decreased from 303.69 mg/L to 0.52 mg/L, which was 99.83% removal efficiency. However, the backward lag phase was found in 0–44 h, with a final biomass of 0.71, which indicated that strain RAD-2 might be more adaptable under a nitrate condition. However, the removal rates of 6.47 mg/(L·h) and 6.32 mg/(L·h) were detected for strain RAD-2 when nitrate or nitrite was used as the sole nitrogen source, respectively. It should be noted that the maximum nitrite-removal rate of strain RAD-2 that could be achieved was 56.20 mg/(L·h) at 44–48 h of the logarithmic growth phase (Panel B).

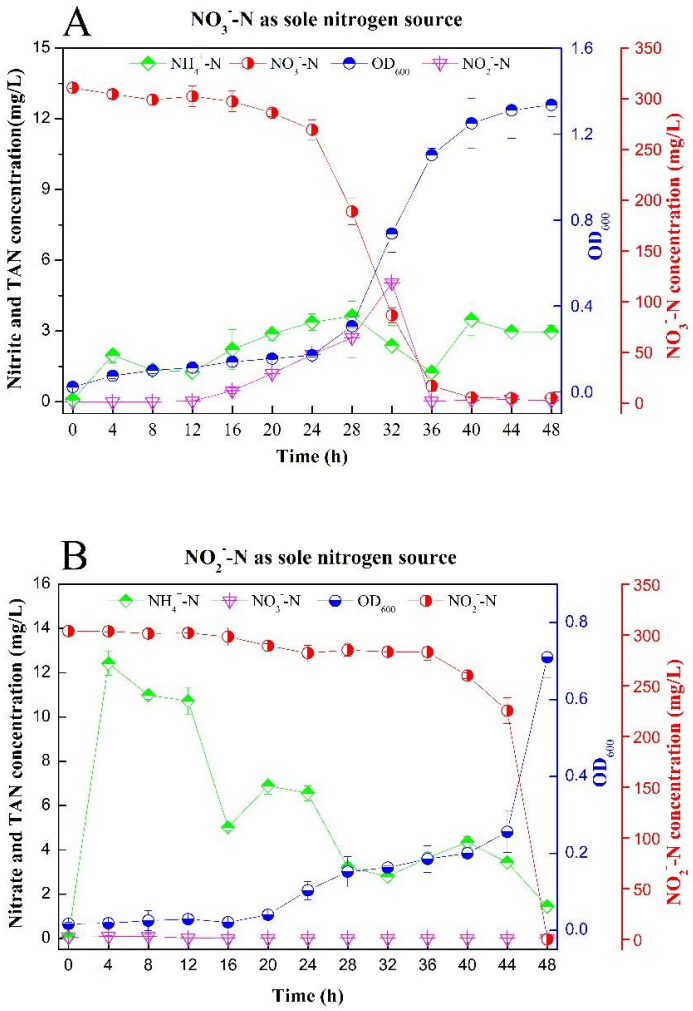

Figure 2. Aerobic nitrogen-removal characteristics and cell growth of strain RAD-2 in denitrification media (DM). (**A**) Nitrate as the sole nitrogen source; and (**B**) nitrite as the sole nitrogen source. Data shown are mean ± SD (error bars) from three replicates.

The heterotrophic nitrification performance of the strain RAD-2 is illustrated in Figure 3. After 48 h of incubation, TAN concentration decreased slightly from the initial 89.64 mg/L to the final 80.73 mg/L, which indicated only 9.94% removal efficiency. No nitrite accumulation was found at any period, while around 0.70 mg/L nitrate was produced. Biomass built up to 0.16 after incubation, which indicated poor growth performance. Therefore, the strain RAD-2 was found to have no heterotrophic nitrification ability under current conditions.

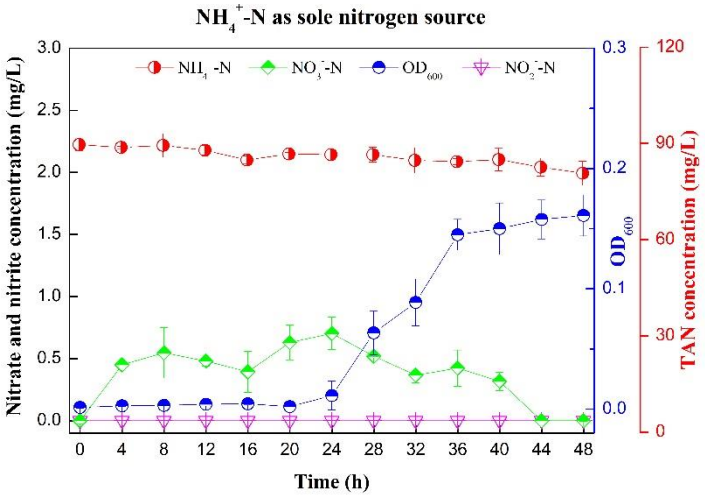

Figure 3. Aerobic ammonium-removal characteristics and cell growth of strain RAD-2 in heterotrophic nitrification media (HNM). Data shown are mean ± SD (error bars) from three replicates.

3.2.2. Single-Factor Experiments of Strain RAD-2

The effects of several environmental factors on the aerobic denitrification performance of strain RAD-2 are shown in Table 2. Aerobic denitrification efficiency relied on the amount of the carbon source, which served as electron donor and energy source. In this study, C/N ratio 5:10 was found optimal for strain RAD-2, having more than 95% nitrate-removal efficiency. A low C/N ratio of 2 lowered nitrate-removal efficiency to 33.59% and had inadequate cell growth, with a final OD_{600} of 0.32 after 48 h incubation. It should be noted that excess C/N ratio also led to a decrease in denitrification performance. On a C/N ratio of 20, only 29.80% nitrate-removal efficiency was achieved, with a final cell growth value of 0.42.

In general, denitrification performance is typically sensitive to temperature variations due to the differences in bacteria species. In this study, strain RAD-2 presents a mesophilic characteristic in the aerobic denitrification process. When the temperature range was 2–15 °C, notably low nitrate-removal efficiency of less than 10% was obtained. Increased temperature could significantly improve denitrification performance, as near as 100% nitrate-removal efficiency, which was gained in the 25–40 °C range.

The different rotation speeds that presented the DO effects on denitrification efficiency were also tested. In this study, strain RAD-2 gained ideal nitrate-removal efficiency in rotation speeds of 100 and 150, which is the equivalent of DO centration at 5.55 and 6.23 mg/L, respectively. Otherwise, only 54.41% nitrate-removal efficiency was obtained under no rotations (DO 0.82 mg/L). In addition, 200 rpm (DO 7.2 mg/L) slightly decreased nitrate-removal efficiency to 89.90%.

Table 2. Effects of varied single factors on the aerobic denitrification performance of strain RAD-2 after 48 h of incubation.

Factor	Variations	Growth (OD600)	Initial Nitrate Concentration (mg/L)	Final Nitrate Concentration (mg/L)	Removal Efficiency (%)
C/N Ratio	2	0.32 ± 0.03	306.33 ± 0.95	203.44 ± 13.05	33.59 ± 4.17
	5	0.79 ± 0.13	305.25 ± 0.65	4.06 ± 0.58	98.67 ± 0.19
	10	1.34 ± 0.05	305.21 ± 0.31	6.48 ± 1.93	97.88 ± 0.63
	15	0.94 ± 0.18	304.02 ± 0.26	40.72 ± 5.77	86.61 ± 1.90
	20	0.42 ± 0.05	304.67 ± 0.45	213.90 ± 12.26	29.80 ± 3.92
Temperature (°C)	5	0.012 ± 0.06	306.37 ± 0.36	305.74 ± 0.40	0.21 ± 0.25
	10	0.11 ± 0.03	305.38 ± 0.35	298.75 ± 2.59	2.17 ± 0.85
	15	0.24 ± 0.06	304.01 ± 0.38	274.22 ± 6.48	9.79 ± 2.25
	25	1.12 ± 0.12	303.29 ± 0.17	6.23 ± 3.61	97.95 ± 1.19
	40	1.02 ± 0.19	304.52 ± 0.37	10.18 ± 1.18	96.66 ± 0.39
Rotation Speed (rpm)	0	0.52 ± 0.11	304.86 ± 0.50	138.99 ± 17.06	54.41 ± 5.53
	50	0.67 ± 0.05	303.62 ± 0.30	92.14 ± 11.05	69.65 ± 3.61
	100	0.95 ± 0.13	305.34 ± 0.21	24.38 ± 6.33	92.02 ± 2.08
	150	1.25 ± 0.11	305.38 ± 0.24	7.02 ± 1.51	97.70 ± 0.49
	200	1.03 ± 0.07	304.43 ± 0.27	30.45 ± 8.37	89.90 ± 2.75

3.3. Expression of Denitrifying Genes by RT-qPCR Analysis

The expression of key denitrifying genes in the aerobic denitrification of strain RAD-2 is shown in Figure 4. On a transcriptional level, the *napA* gene showed the highest expression level in this study, which indicated the aerobic denitrification characteristic of strain RAD-2. The *nirS* and *nosZ* genes had similar expression intensity, which was one order of magnitude lower than that of the *napA* gene. Moreover, the *norB* gene was found to have significantly low expression during the whole period, and its intensity was negligible when compared with other genes (*napA*, *nirS*, and *nosZ*). All genes showed a decrease or low expression intensity during 0–24 h. Then, notable synergetic expressions of *napA*, *nirS*, and *nosZ* genes showed an increase in the range 24–36 h. It should be noted that though maximum expression intensity was found at 0 h, this time point should reflect the transcriptional state of strain RAD-2 in LB media, as we obtained the samples immediately after the inoculation.

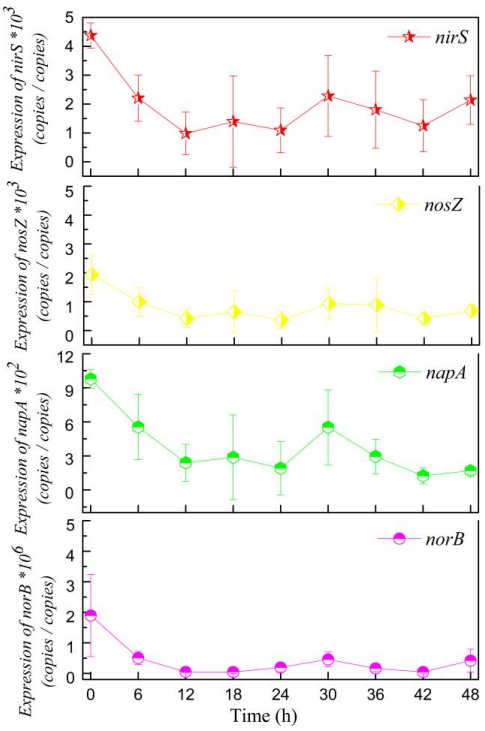

Figure 4. Aerobic denitrifying gene expression of strain RAD-2 during 48 h incubation.

4. Discussion

4.1. Characteristics and Identification

In this study, *Marinobacter hydrocarbonoclasticus* strain RAD-2 was isolated from a denitrifying reactor using PHBV as the carbon source and biofilm carrier. In general, *Marinobacter hydrocarbonoclasticus* is the species of the genus *Marinobacter*, which belongs to the class Gammaproteobacteria. Species of this genus are Gram-staining-negative, rod-shaped, and motile [31]. A notable feature of *Marinobacter hydrocarbonoclasticus* is the utilization of various hydrocarbons as sole carbon and energy sources [32]. For example, using waste frying oil as the inducer carbon source, the produced biosurfactant of the strain *Marinobacter hydrocarbonoclasticus* SdK644 could be applied to improve crude-oil solubilization in a marine environment [33]. Therefore, strain RAD-2 might have the ability to use biodegradable polymers (PHBV etc.) for denitrification.

Based on the 16S rRNA gene sequences, strain RAD-2 formed a distinct branch with strain ATCC49840 and strain VT8, and this clade was close to the groups containing *Marinobacter* sp. NN5, *Marinobacter* sp. U1369-101122-SW163, and *Marinobacter hydrocarbonoclasticus* strain NY-4. However, the genus *Marinobacter* was reported to have many different phenotypic characteristics in the denitrification process. For example, strain RAD-2, *Marinobacter* sp. NN5, and *Marinobacter* sp. F6 were found to have efficient aerobic denitrification ability [20,25], while *Marinobacter hydrocarbonoclasticus* strain NY-4 only had anaerobic denitrification ability [34].

4.2. Nitrogen-Removal Performance Evaluation

In this study, strain RAD-2 presented efficient aerobic denitrification performance. An average removal rate of 6.47 mg/(L·h) and 6.32 mg/(L·h) was found in strain RAD-2 when nitrate or nitrite was used as the sole nitrogen source, respectively (Figure 2). This was much faster than several other *Marinobacter* strains. For example, *Marinobacter* sp. NN5 and *Marinobacter* sp. F6 were reported to have a 4.7 mg/(L·h) and 1.46 mg/(L·h) NO_3^--N removal rate, respectively [20,25]. In other genera, *Bacillus methylotrophicus* L7 was found to have a 5.81 mg/(L·h) NO_2^--N removal rate [35]. *Pseudomonas migulaer* AN-1 has a 1.57 NO_3^--N mg/(L·h) or 0.69 NO_2^--N mg/(L·h) removal rate [36]. *Pseudomonas putida* Y-12 has a 1.57 NO_3^--N mg/(L·h) or 1.60 NO_2^--N mg/(L·h) removal rate [37]. Otherwise, strain RAD-2 cannot perform heterotrophic nitrification, which was consistent with *Marinobacter* sp. NN5 [20]. Only *Marinobacter* sp. F6 was reported to have the simultaneous ability of heterotrophic nitrification and aerobic denitrification in the *Marinobacter* genus [25].

Based on several single-factor experiments, strain RAD-2 showed good ecological width in marine-aquaculture conditions. A 5:10 C/N ratio, 25–40 °C temperature, and 100–150 rpm rotation speed were the optimal conditions for aerobic denitrification (Table 2). It is reported that *Marinobacter* sp. NN5 has 35 °C temperature, 6:8 C/N ratio, and 150 rpm rotation speed as optimal conditions [20]. Therefore, strain RAD-2 could adapt to a lower temperature of 25 °C, which might increase its application in marine aquaculture, as temperatures 25–35 °C are the optimal environmental conditions for most cultured species. However, to better use the strain in practice, toxicology research should also be performed for strain RAD-2 in the future [38,39].

4.3. Aerobic Denitrification Pathways Analysis

The expression of key denitrifying genes in the aerobic denitrification of strain RAD-2 is shown in Figure 4. In general, aerobic denitrification has two different electron-transfer pathways [22]. The expression of the *napA* gene can guarantee that the aerobic denitrification strain still has electron-transfer capacity under aerobic conditions [22]. In anoxic denitrification, electron transfer to nitrate can be blocked as encoding gene *narG* is sensitive to oxygen [13]. In this study, the *napA* gene had a maximum expression level, which was responsible for the efficient aerobic nitrate removal performance. The synergetic expressions of the *napA*, *nirS*, and *nosZ* genes increased during 24–36 h, which resulted in strain growth and nitrate elimination. (Figure 2A). It should also be noted that

the *norB* gene showed very low expression (Figure 4). The *norB* gene was in charge of nitric oxide reductase production [22], which transfers NO to N_2O. N_2O emission has recently been attracting more attention due to its environmental impact [40]. In the *Marinobacter* genus, many strains were reported as having zero N_2O emissions. For example, *Marinobacter* sp. NN5 has total N_2 production without N_2O in aerobic conditions, while *Marinobacter hydrocarbonoclasticus* strain NY-4 was reported to produce no N_2O in anaerobic conditions [20,34]. Conventionally, the high activity of nitrous oxide reductase, which was encoded by the *nosZ* gene, was charged with the efficient transfer of N_2O to N_2. Here, we also give molecular evidence that the *Marinobacter hydrocarbonoclasticus* RAD-2 strain has little expression intensity, which might be another reason for its zero N_2O emissions. Since N_2O is an important greenhouse gas, and aquaculture systems are considered an important anthropogenic source of N_2O emission [41], strain RAD-2 might have great potential for aerobic denitrification in marine RAS applications.

5. Conclusions

An aerobic denitrifier strain was isolated from a long-term PHBV-supported denitrification reactor that was operated under alternate aerobic/anoxic conditions. The strain was identified as *Marinobacter hydrocarbonoclasticus* RAD-2 based on 16S rRNA-sequence phylogenetic analysis. Strain RAD-2 showed high efficiency for aerobic denitrification when using NO3$^-$-N or NO_2^--N as the sole nitrogen source, while almost being unable to perform heterotrophic nitrification. The average NO_3^--N and NO_2^--N removal rates were 6.47 mg/(L·h) and 6.32 mg/(L·h), respectively. Single-factor experiments indicated that a 5:10 C/N ratio, 25–40 °C temperature, and 100–150 rpm rotation speed were the optimal conditions for aerobic denitrification. Furthermore, the denitrifying gene *napA* had maximum expression intensity on a transcriptional level, followed by *nirS* and *nosZ*. The *norB* gene was found to have significantly low expression during the whole period. Therefore, the denitrifying pathways showed its aerobic denitrification characteristic and potentially fewer N_2O emissions. Overall, the efficient aerobic denitrification performance of strain RAD-2 makes it a potential candidate for bioaugmentation to improve the effluent treatment of marine RAS.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4441/10/10/1298/s1, Figure S1: Scanning electron microscope micrograph of *Marinobacter hydrocarbonoclasticus* strain RAD-2, Table S1: PCR primers used of *16s rRNA*, *napA*, *nirS*, *norB* and *nosZ* for strain RAD-2.

Author Contributions: Conceptualization: D.K., Y.R. and F.L.; data curation: W.L. and Y.D.; investigation: D.K., W.L., G.C. and J.Y.; writing—original draft: D.K. and Y.R.; writing—review and editing: Y.R.

Funding: This research was funded by Natural Science Fund of China grant number [31402348 and 41401556], Key Research and Development Projects of Zhejiang Province grant number [2018C02037] and Agriculture Technology Extension Funds of Zhejiang University (2017). The APC was funded by Key Research and Development Projects of Zhejiang Province [2018C02037].

Acknowledgments: We thank the reviewers for their insightful comments and suggestions.

Conflicts of Interest: The authors declare no conflict of interest.

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