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REVIEW
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Advances in Research Into and Applications of Heterotrophic Nitrifying and Aerobic Denitrifying Microorganisms

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With the increasing use of animal and plant proteins, pollution due to nitrogen sources is attracting increasing attention. In particular, the amount of nitrogen-containing sewage discharged into the environment has increased significantly, causing eutrophication of water bodies and environmental degradation of water quality. Traditionally, nitrifying bacteria perform ammonia nitrification under aerobic conditions, while denitrifying bacteria perform nitrate/nitrite denitrification under anaerobic conditions. However, heterotrophic nitrifying and aerobic denitrifying microorganisms (HNADs) perform ammonia nitrification and nitrate/nitrite denitrification under the same aerobic conditions using an organic carbon source, which is a much simpler and more efficient process. In this review, the distribution and evolutionary relationships of novel HNADs strains are presented, and the influencing factors, metabolic pathways, key enzymes, and practical applications of HNADs are reviewed.

Keywords: heterotrophic nitrifying, aerobic denitrifying, nitrogen biotransformation pathway, influencing factors, HNADs

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1 INTRODUCTION

With the increasing use of animal and plant proteins in the farming industry, pollution due to nitrogen sources is attracting increasing attention. In particular, the amount of nitrogen-containing sewage discharged into the environment has increased significantly, and this has led to various ecological health and environmental safety issues, such as the eutrophication of water bodies (Zhu et al., 2012). Eutrophication causes algal blooms that threaten the ecosystem function of freshwater, aquaculture safety, and public health, and it is becoming a major environmental problem (Tang et al., 2019). Therefore, treating nitrogen-containing wastewater has become an important element of environmental management related to sustainable development of the environment.

Conventionally, high-concentration nitrogen-containing wastewater is treated using physical, chemical, or biological methods (Zhang et al., 2011). The physical method mainly uses physical adsorption, which only adsorbs ammonia nitrogen and nitrite in the water body on the solid surface, and does not fundamentally reduce the content of ammonia nitrogen and nitrite in the water body. Chemical method in the use of the same high cost, small range, low security, and other disadvantages. Biological method mainly uses nitrifying bacteria and denitrifying bacteria to convert ammonia nitrogen and nitrite into nitrogen gas through nitrification and denitrification, because the reaction

TABLE 1 | Distribution and domains of HNADs.

Domain	Strain	Isolation environment	Nitrogen source(s)	References
Prokaryote	*Arthrobacter arilaitensis* Y-10	Soil	Ammonium, nitrate	He et al. (2017)
	Acinetobacter sp. ND7	Sludge	Ammonium, nitrate, nitrite	Xia et al. (2020)
	Acinetobacter sp. FYF8	Poor-nutrient ecosystem	Nitrate	Fan et al. (2021)
	Acinetobacter sp. YS2	Wastewater	Ammonium, nitrate, nitrite	Lang et al. (2020)
	Acinetobacter sp. H36	Lake	Nitrate	Su et al. (2017)
	Acinetobacter sp. JR1	Raw water	Ammonium, nitrate, nitrite	Yang et al. (2019)
	Acinetobacter sp. Y16	Raw water	Ammonium	Huang et al. (2013)
	Acinetobacter junii YB	Sludge	Ammonium, nitrate, nitrite	Ren et al. (2014)
	Acinetobacter calcoaceticus HNR	Bioreactor	Ammonium	Zhao et al. (2010)
	Acinetobacter baumannii AL-6	Sludge	Ammonium	An et al. (2020)
	Acinetobacter sp. T1	Sludge	Ammonium, nitrate, nitrite	Chen et al. (2019)
	Achromobacter sp. GAD3, *Comamonas* sp. GAD4	Landfill leachate	Ammonium	Chen and Ni (2010)
	Aeromonas sp. HN-02	Activated sludge	Ammonium, nitrate, nitrite	Chen et al. (2014)
	Agrobacterium sp. LAD9	Landfill leachate	Ammonium, nitrate, nitrite	Chen and Ni (2012)
	Alcaligenes sp. TB	Biofilter	Ammonium, nitrate	Chen et al. (2016)
	Alcaligenes faecalis sp. No. 4	Sewage sludge	Ammonium	Joo et al. (2005)
	Alcaligenes faecalis SDU20	Swine	Ammonium	Chen et al. (2021)
	Anoxybacillus contaminans HA	Treatment system	Ammonium, nitrate	Chen et al. (2015)
	Bacillus cereus GS-5	Bio-film sample	Ammonium, nitrate, nitrite	Rout et al. (2017)
	Bacillus litoralis N31	Mariculture water	Ammonium, nitrate, nitrite	Huang F. et al. (2017)
	Bacillus methylotrophicus L7	Wastewater	Ammonium, nitrate	Zhang et al. (2012)
	Bacillus simplex H-b	Soil	Ammonium, nitrate, nitrite	Yang Q. et al. (2021)
	Bacillus sp. K5	Bio-trickling filter	Ammonium	Yang et al. (2017)
	Bacillus subtilis A1	Wastewater	Ammonium	Yang et al. (2011)
	Diaphorobacter sp	Wastewater	Ammonium	Khardenavis et al. (2007)
	Enterobacter huaxiensis Z1, *Klebsiella pneumoniae* Z2	Sediment	Ammonium, nitrate, nitrite	Zhang Y. et al. (2019)
	Enterobacter cloacae HNR	Sludge	Nitrate	Guo et al. (2016)
	Halomonas alkaliphile HRL-9	Seawater biofilter	Nitrate	Ren et al. (2019)
	Klebsiella pneumoniae CF-S9	Wastewater	Ammonium, nitrate, nitrite	Padhi et al. (2013)
	Klebsiella pneumoniae, Klebsiella variicola, Klebsiella variicola	Sludge	Ammonium, nitrate, nitrite	Feng et al. (2018)
	Klebsiella sp. TN-10	Wastewater	Ammonium	Li et al. (2019)
	Marinobacter NNA5	Biofilter	Nitrate, nitrite	Liu et al. (2016)
	Paracoccus denitrificans DYTN-1	Sludge	Total nitrogen	Zhao et al. (2020)
	Paracoccus denitrificans Z195	Sludge	Total nitrogen	Zhang et al. (2020)
	Photobacterium sp. NNA4	Recirculating aquaculture system	Ammonium, nitrate, nitrite, hydroxylamine	Liu et al. (2019)
	Pseudomonas stutzeri T13	Sludge	Ammonium	Sun et al. (2017), Li et al. (2012)
	Pseudomonas sp. GZWN4	Pond	Ammonium, nitrate, nitrite	Su et al. (2021)
	Pseudomonas balearica RAD-17	Reactor	Nitrate, nitrite	Ruan et al. (2020)
	Pseudomonas stutzeri D6	Sludge	Ammonium, nitrite	Yang et al. (2012)
	Pseudomonas sihuiensis LK-618	Sediment	Ammonium, nitrate, nitrite	Hong et al. (2021)
	Pseudomonas tolaasii Y-11	Soil	Ammonium, nitrate, nitrite	He et al. (2016)
	Pseudomonas stutzeri ZF31	Drinking water	Nitrate	Huang et al. (2015)
	Pseudomonas stutzeri YG-24	Lake	Ammonium, nitrate, nitrite	Li et al. (2015)
	Pseudomonas stutzeri TR2 and K50	Pond, wastewater, and soil	Nitrate, nitrite	Takaya et al. (2003)
	Pseudomonas mendocina TJPU04	Sludge	Ammonium, nitrate, nitrite	He X. et al. (2019)
	Pseudomonas aeruginosa P-1	Sewage sludge	Ammonium, nitrate, nitrite	Wei et al. (2021)
	Pseudomonas chloritidismutans K14	Pond	Ammonium	Hou et al. (2021)
	Pseudomonas mendocina X49	Sewage	Ammonium, nitrite	Xie et al. (2021)
	Pseudomonas stutzeri YZN-001	Pig manure effluent	Nitrate, nitrite	Zhang et al. (2011)
	Pseudomonas stutzeri GEP-01	Sludge	Ammonium	Gao et al. (2020)
	Pseudomonas stutzeri XL-2	Wastewater	Nitrate	Zhao et al. (2018)
	Rhodococcus sp. CPZ24	Wastewater	Ammonium, nitrate	Chen et al. (2012)
	Serratia marcescens W5	Lake	Ammonium	Wang et al. (2016)
	Serratia marcescens CL1502	Deep-sea sediment	Ammonium, nitrate, nitrite	Huang G. et al. (2017)
	Vibrio sp. Y1-5	Sediment	Ammonium, nitrate	Li et al. (2017)
	Vibrio sp. AD2	Biofilter	Nitrate	Ren et al. (2021)
	Zobellella denitrificans A63	CW system	Ammonium, nitrate	Fu et al. (2019)
Eukaryote	*Barnettozyma californica* K1	Sediment	Ammonium, nitrate, nitrite	Fang et al. (2021)
	Fusarium solani RADF-77	Reactor	Nitrate	Cheng et al. (2020)
	Hanseniaspora uvarum KPL108	Sediment	Nitrate	Zhang et al. (2018b)

(Continued on following page)

TABLE 1 | (*Continued*) Distribution and domains of HNADs.

Domain	Strain	Isolation environment	Nitrogen source(s)	References
	Penicillium tropicum IS0293	Lake	Ammonium, nitrate, nitrite	Yao et al. (2020)
	Sporidiobolus pararoseus Y1	Marine aquaculture water	Ammonium, nitrate, nitrite	Zeng et al. (2020)

conditions are mild and can reduce the total nitrogen content in the water body, and is the safest and most effective method (Khardenavis et al., 2007). In particular, heterotrophic nitrifying and aerobic denitrifying microorganisms (hereinafter referred to as HNADs) have been studied increasingly since *Thiosphaera pantotropha* was isolated in the 1980s (Robertson and Kuenen, 1983), and these include *Acinetobacter* (An et al., 2020), *Alcaligenes* (Chen et al., 2021), *Arthrobacter* (He et al., 2017), *Klebsiella* (Padhi et al., 2013), *Bacillus* (Rout et al., 2017), *Enterobacter* (Guo et al., 2016), *Paracoccus* (Zhang et al., 2018a), and *Pseudomonas* (He et al., 2016). Heterotrophic nitrifying and aerobic denitrifying microorganisms (HNADs) can use organic matter as a carbon source to oxidize ammonia to nitrite and nitrate, while converting nitrate and nitrite to gaseous nitrogen under aerobic conditions, resulting in both nitrification and denitrification (He X. et al., 2019). Compared with the conventional autotrophic nitrification and anaerobic denitrification processes, HNADs are more streamlined and offer smaller reactor size, lower cost, and higher nitrogen-removal efficiency (Duan et al., 2015).

Heterotrophic nitrification–aerobic denitrification is a biological nitrogen removal technology with unique advantages compared with traditional anaerobic denitrification and autotrophic aerobic denitrification proposed in recent years. On the one hand, denitrification is carried out under aerobic conditions, nitrification and denitrification can be carried out simultaneously in one reactor, and equipment and operation costs are significantly reduced; on the other hand, compared with the traditional autotrophic nitrifying bacteria, heterotrophic nitrifying bacteria have a short generation cycle and rapid growth, which can be applied to the environment of organic wastewater. The products of nitrification can be directly used as substrate for denitrification, which avoids the inhibition of nitrification and strengthens the nitrification and denitrification processes.

This review summarizes the recent research on HNAD strains and their physiological characteristics and metabolic pathways. The distribution and evolutionary relationship of novel HNAD strains are presented, and the influencing factors, metabolic pathways, and critical enzymes of HNADs are reviewed.

2 DISTRIBUTION AND EVOLUTIONARY RELATIONSHIPS OF NOVEL HNAD STRAINS

Recently, new advances have been made in studying HNADs from various environmental sources, and many new microbial strains have been screened and identified. The domain, strain, nitrogen source, and isolation environment of each of these reported HNADs are given in **Table 1**. HNADs are found mostly in soil, sludge, wastewater, and lakes because these environments contain abundant nitrogen sources. However, despite different HNADs being isolated from different environments, their HNAD function is the same. The reported HNADs include not only bacteria but also fungi and yeasts, and their scope is thus relatively wide. **Figure 1** shows partial 16S rRNA sequences of HNAD bacteria downloaded from GenBank, with their evolutionary relationship tree plotted.

Compared to traditional denitrifying bacteria that can only use nitrate, many HNADs can also use ammonia and/or nitrite and convert them to gaseous nitrogen and biomass through the aerobic denitrification pathway and assimilation process (Chen and Ni, 2012; Xia et al., 2020). Under anaerobic conditions, denitrifying bacteria usually use nitrate as the final electron acceptor, but HNADs use oxygen as the final electron acceptor, thereby replacing nitrate for electron transfer in the aerobic respiratory chain and generating more energy. Therefore, HNADs are better in terms of growth rate and nitrogen-removal efficiency. When *Pseudomonas tolaasii* Y-11was cultivated with an initial nitrate concentration of over 200 mg/L, 93.5% of the nitrate was removed in 4 days without nitrite accumulation via the denitrification pathway (He et al., 2016). In *Pseudomonas stutzeri* ZF31, calculated by nitrogen balance, the results showed that about 75% of the initial nitrate was converted to gaseous nitrogen (Huang et al., 2015). Ammonia nitrogen can also be converted to gaseous nitrogen via the denitrification pathway by HNADs. For example, *Acinetobacter* sp. JR1 can use ammonium nitrogen as the sole nitrogen source for conversion into gaseous nitrogen under aerobic conditions (Yang et al., 2019). Similar results were reported for *P. stutzeri* YZN-001 (Zhang et al., 2011), *Acinetobacter* sp. YS2 (Lang et al., 2020), and mixed *Bacillus* strains (Kim et al., 2005). In addition, some HNADs such as *Rhodococcus erythropolis* Y10 (Ma et al., 2021) remove ammonia nitrogen by assimilation, converting the nitrogen source into biological nitrogen for their own growth.

3 NITROGEN AND AMMONIUM BIOTRANSFORMATION PATHWAYS IN HNADS

3.1 Nitrogen Biotransformation Pathways in HNADs

3.1.1 Ammonium Biotransformation Pathway in HNADs

The conventional ammonium nitrification pathway is coordinated by two types of bacteria: (i) ammonia-oxidizing bacteria, which are responsible for oxidizing ammonium to nitrite; and (ii) nitrite-

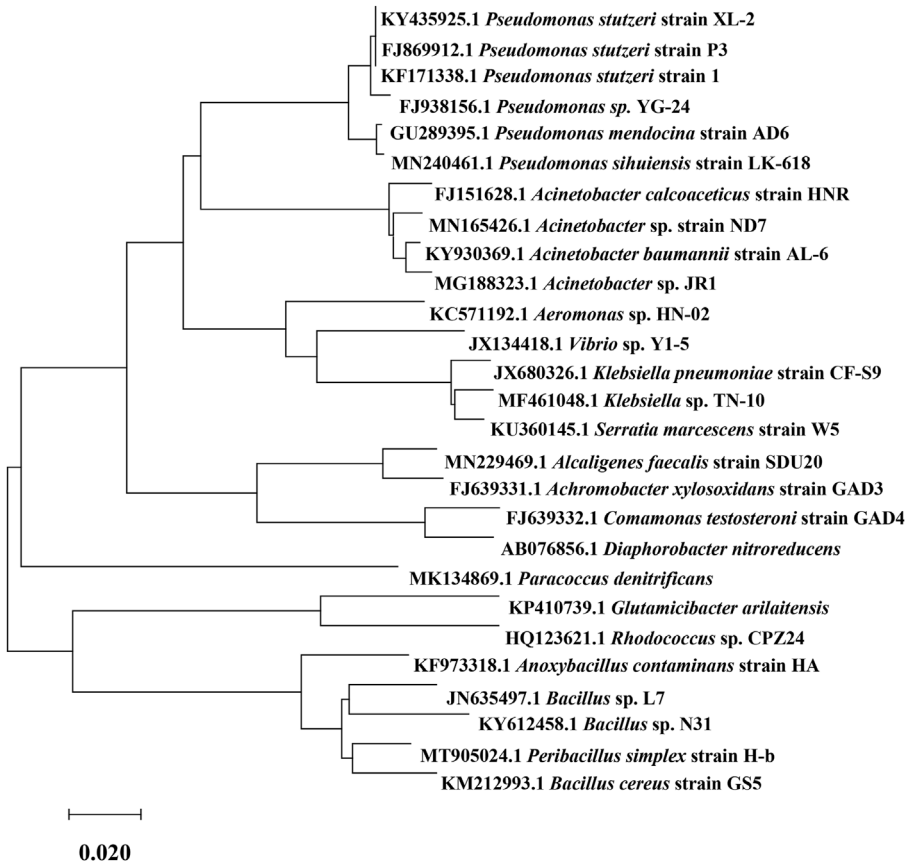

FIGURE 1 | Evolutionary relationship tree of heterotrophic nitrifying and aerobic denitrifying (HNAD) bacteria.

oxidizing bacteria, which are responsible for oxidizing nitrite to nitrate. Nitrate is finally converted to gaseous nitrogen by heterotrophic denitrifying bacteria via the denitrification pathway under anaerobic conditions (Jaroszynski and Oleszkiewicz, 2011). The conversion efficiency of HNADs is better than that of the conventional process of ammonium removal because there are differences in intermediate products and key catalytic enzymes.

In the 1990s, a classical coupled model of ammonium nitrification in HNAD bacteria was reported (Wehrfritz et al., 1993). Ammonium is oxidized to hydroxylamine in the periplasm by ammonia monooxygenase (AMO), followed by oxidation to nitrite by hydroxylamine oxidoreductase (HAO), and finally to nitrate in the cytoplasm (Song et al., 2021). In HNADs, nitrate is reduced via denitrification by a series of oxidoreductases under aerobic conditions, and stable isotopes and enzyme inhibitors are used for more-accurate detection of intermediate metabolites in the study of nitrogen conversion pathways in HNADs.

Alcaligenes faecalis NR can convert ammonium to N_2O and N_2 with ammonium as the sole nitrogen source, and intermediate nitrate and nitrite are not detected under aerobic conditions (Zhao et al., 2012). When hydroxylamine is the sole nitrogen source, gaseous nitrogen is still detected, but only hydroxylamine oxidase activity is detected, and nitrate and nitrite reductase activity is undetected (Zhao et al., 2012). Hydroxylamine is converted directly to nitrous oxide replacing nitrite, and a similar finding has been reported for *Acinetobacter calcoaceticus* HNR (Zhao et al., 2010). It has been speculated that the ammonium-removal pathway of *A. calcoaceticus* HNR and *A. faecalis* NR is NH_4^+-N→NH_2OH→N_2O→N_2 (a shortcut ammonium metabolic pathway), and experiments have confirmed that *Photobacterium* sp. NNA4 can transform hydroxylamine directly to N_2O when hydroxylamine is the sole nitrogen source (Liu et al., 2019).

Enzyme activation inhibitors can block metabolic pathways, resulting in the accumulation of intermediate products or the disappearance of end products, and they are often used to study the metabolic pathways of nitrogen conversion. In *Alcaligenes* sp. TB, when nitrite reductase inhibitor (pb^{2+}) is present, the accumulation of nitrate and nitrite is detected, but gaseous nitrogen is not detected, whereas when nitrate reductase inhibitor (Na_2WO_4) is present, the accumulation of nitrate and gaseous nitrogen is detected simultaneously; these results show that the strain TB carries out ammonium removal via the pathway NH_4^+-N→NH_2OH→NO_2^--N→N_2O→N_2 (an incomplete ammonium metabolic pathway) (Chen et al., 2016). The production of gaseous nitrogen is found to be accompanied by the interconversion of nitrite and nitrate, which has similarly been reported for *Agrobacterium* sp. LAD9

(Chen and Ni, 2012). In addition, *Alcaligenes* sp. TB can convert approximately 35.7% of the ammonium to gaseous nitrogen without enzyme inhibitors via another pathway, that is, NH_4^+-N→NH_2OH→NO_2^--N→NO_3^--N→NO_2^--N→N_2O→N_2 (a complete ammonium metabolic pathway) (Chen et al., 2016). The same nitrogen-removal pathway has been reported for *B. subtilis* A1 (Yang et al., 2011), *K. pneumoniae* CF-S9 (Padhi et al., 2013), *B. cereus* GS-5 (Rout et al., 2017), and *Rhodococcus* sp. CPZ24 (Chen et al., 2012).

Different nitrogen-source combinations also affect the denitrification pathway of ammonium. When ammonium and nitrite are present simultaneously, the ammonium is transformed directly to gaseous nitrogen via a shortcut ammonium metabolic pathway, but when ammonium and nitrate are present simultaneously, the ammonium is first transformed to nitrate and then reduced to gaseous nitrogen via a complete ammonium metabolic pathway (Wei et al., 2021). Furthermore, compared with the traditional autotrophic nitrification pathway, the hydroxylamine pathway is simpler, which makes it more advantageous to use HNADs in treating high-concentration nitrogenous wastewater (Peng and Zhu, 2006).

3.1.2 Nitrate, Nitrite, and Nitrous Oxide Biotransformation Pathways in HNADs

The reduction of nitrate to gaseous nitrogen by HNADs is accomplished via four reductases under aerobic conditions in four processes, that is, NO_3^--N→NO_2^--N→NO→N_2O→N_2 (Song et al., 2021). Nitrite reductase is also a bottleneck in the denitrification pathway of HNADs because it is as sensitive to oxygen as are traditional anaerobic denitrifying bacteria. The ability of *P. stutzeri* T13 to convert nitrate efficiently with nitrate as the sole nitrogen source has been investigated, finding that the conversion efficiency of intermediate nitrite metabolites is significantly higher at low levels of dissolved oxygen (DO) than at high levels (Sun et al., 2015). This sensitivity to oxygen inhibits the reduction of nitrite, and it accumulates during denitrification with high DO levels. Some HNAD bacteria have different properties and are effective in converting nitrite to gaseous nitrogen at high DO levels, such as *P. stutzeri* ZF31, *P. mendocina* X49, and *Vibrio* sp. AD2 (Huang et al., 2015; Ren et al., 2021; Xie et al., 2021).

Nitrite is an intermediate product of the denitrification metabolic pathway, and because of its cytotoxicity, few HNADs can use nitrite directly for denitrification conversion. It has been reported that *Pseudomonas* sp. yy7 can use nitrite via assimilation and denitrification (Wan et al., 2011), and isotope-tracking experiments have shown that *P. stutzeri* converts nitrous oxide (N_2O)—a precursor of denitrification—to nitrogen gas (N_2) under aerobic conditions (Desloover et al., 2014). The denitrification pathway with nitrite or nitrous oxide as the initial nitrogen source is an incomplete metabolic pathway.

3.2 Assimilation and Dissimilation of Nitrogen in HNADs

Assimilation synthesizes proteins and nucleic acids required for microbial growth, which are important for both cell growth and nitrogen conversion. It has been reported that *R. erythropolis* Y10 can use ammonium, nitrate, and nitrite via the assimilation pathway; 98.23% of the nitrogen is assimilated to biomass nitrogen with ammonium as the sole nitrogen source, much higher than the 52.73 and 56.22% for nitrate and nitrite, respectively (Ma et al., 2021). *Vibrio* sp. Y1-5 can assimilate nitrate to biomass nitrogen under aerobic conditions, and the assimilation efficiency increases with increasing DO concentration (Li et al., 2017).

Transcriptomics and the expression of critical enzyme genes have been used to study the assimilation of metabolic pathways in *Klebsiella* sp. KSND when using different nitrogen sources under aerobic conditions, and the results showed that the transcription levels of NADP-glutamate dehydrogenase increased by a factor of approximately 8.6 and glutamine synthetase was not detected. This suggests that NH_4^+-N assimilation is through the NADP-glutamate dehydrogenase pathway in *Klebsiella* sp. KSND (Jin et al., 2019).

A new nitrate-dissimilation conversion pathway (NO_3^--N→NO_2^--N→NH_4^+-N) for *Pseudomonas putida* Y-9 was reported via ^{15}N isotope experiments in which nitrate was reduced to ammonium under aerobic conditions (Huang X. et al., 2020). This pathway occurs under not only aerobic conditions but also anaerobic ones (Yoon et al., 2015).

The nitrogen conversion pathways of HNADs are shown in **Figure 2** and include mainly ammonia nitrification, nitrate denitrification, nitrite incomplete denitrification, and nitrate assimilation/dissimilation under aerobic conditions.

4 CRITICAL ENZYMES OF BIOTRANSFORMATION PATHWAYS IN HNADS

4.1 Critical Enzymes of Denitrification Process

4.1.1 Nitrate Reductase (NR)

The reduction of nitrate to nitrite is the start of the denitrification reaction and is carried out by NR. Outside the cell is periplasmic nitrate reductase (Nap), and bound to the cell membrane is nitrate reductase (Nar) (**Figure 3**) (Vivián et al., 1999).

Nar is a Mo-containing enzyme that has three catalytic subunits: NarG, NarH, and NarI. NarG is the largest catalytic subunit, comprising a [4Fe-4S] center (FS0) and a Moco active site. NarH binds to NarG and is located in the cytoplasm, containing three [4Fe-4S] centers (FS1, FS2, and FS3) and one structurally distinct [3Fe-4S] center (FS4); it is mainly responsible for electron transfer. NarI is the smallest subunit; it is anchored to the cell membrane and is responsible for the transmembrane movement of protons (Coelho and Romao, 2015). The expression of Nar has been detected in *B. cereus* GS-5 under aerobic conditions and was encoded by 561 bp *nar* (Rout et al., 2017).

Nap is an extracellular enzyme that reduces nitrate in the periplasm under aerobic conditions and plays a critical role in HNADs; it was first purified from *Paracoccus pantotrophus* (Sears et al., 1995; Yang J. et al., 2020). Nap may be a key factor for nitrogen conversion by HNAD bacteria under aerobic conditions.

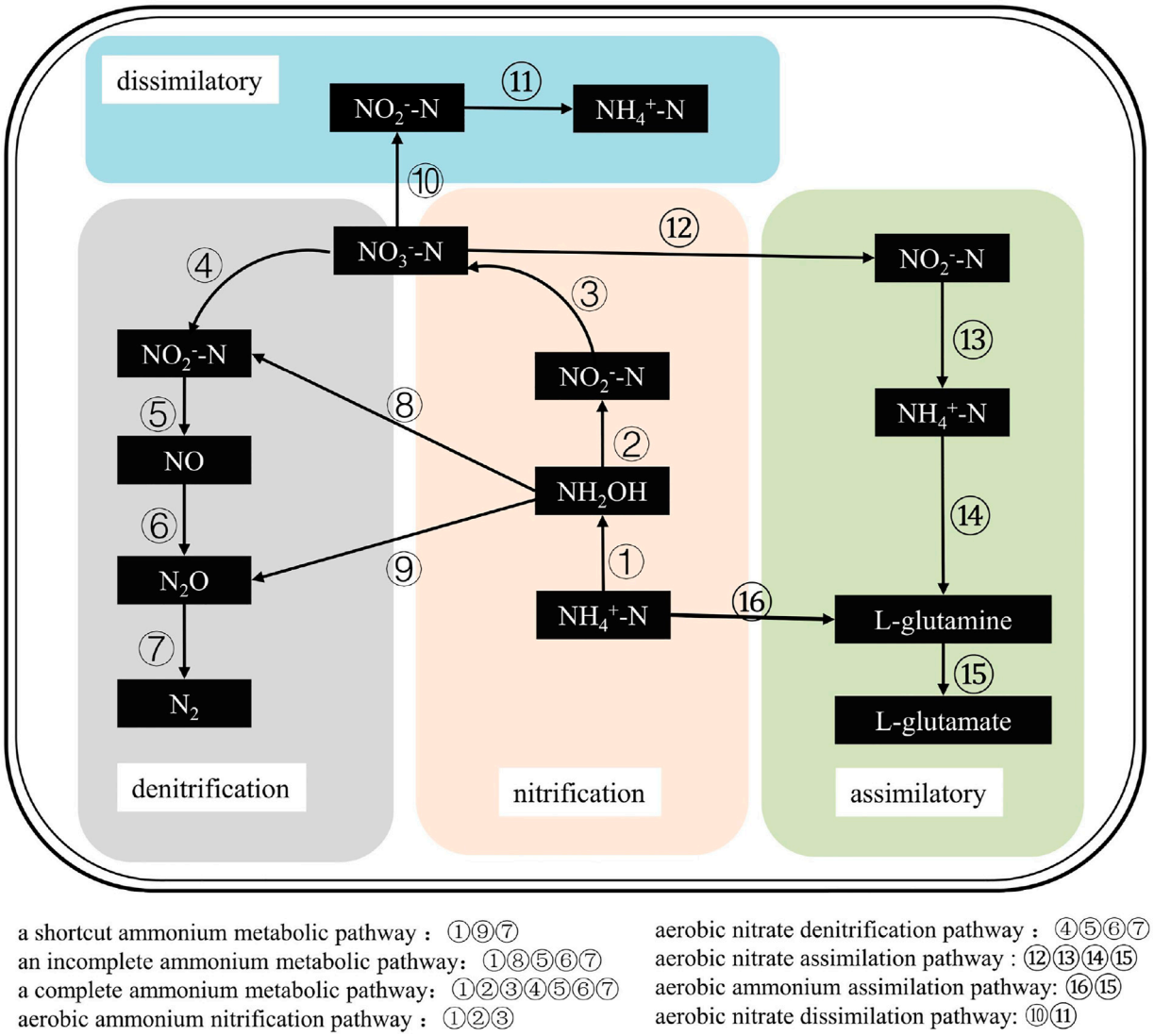

FIGURE 2 | The nitrogen conversion pathways of HNADs, which include mainly ammonia nitrification, nitrate denitrification, nitrite incomplete denitrification, and nitrate assimilation/dissimilation under aerobic conditions.

The first crystal structures of periplasmic nitrate reductases were reported in *Desulfovibrio desulfuricans* at 1.9-Å resolution (Dias et al., 1999). Nap contains two subunits, that is, NapA and NapB, and the genes encoding these are *napA* and *napB*, respectively; *napA* has been successfully amplified in *P. stutzeri* XL-2 (Zhao et al., 2018).

4.1.2 Nitrite Reductase (NIR)

Nitrite is converted to nitric oxide catalyzed via NIR, which contains two types of catalase: cd1Nir and CuNir (**Figure 3**). These two enzymes are nonhomologous isozymes and usually cannot coexist in the same microorganism (Zumft et al., 1994). However, the coexistence of two enzymes has been reported in the same microorganism, such as in *Bradyrhizobium oligotrophicum* S58 (Sanchez and Minamisawa, 2018) and *Rhodothermus marinus* (Graf et al., 2014).

Cd1Nir contains two identical subunits with cofactors heme d1 and heme c; it is a homologous dimer protein and is encoded by *nirS* (Nojiri et al., 2009). In *Agrobacterium* sp. LAD9, 799 bp of the *nirS* gene fragment have been amplified under aerobic conditions (Chen and Ni, 2012).

CuNir is a homologous trimeric protein containing three identical subunits with two Cu-binding sites on each (Godden et al., 1991). CuNir is usually encoded by the *nirK* gene and sometimes by the *nirV* gene (Yang J. et al., 2020). The *nirK* gene of nitrite reductases in *S. pararoseus* Y1 and in *Alcaligenes faecalis* NR has been amplified (Huang et al., 2018; Zeng et al., 2020).

4.1.3 Nitric Oxide Reductase (NOR)

NOR is embedded in the cell membrane with the catalytic site in the periplasm and is responsible for the reduction of nitric oxide to nitrous oxide (**Figure 3**). NOR is divided into three types

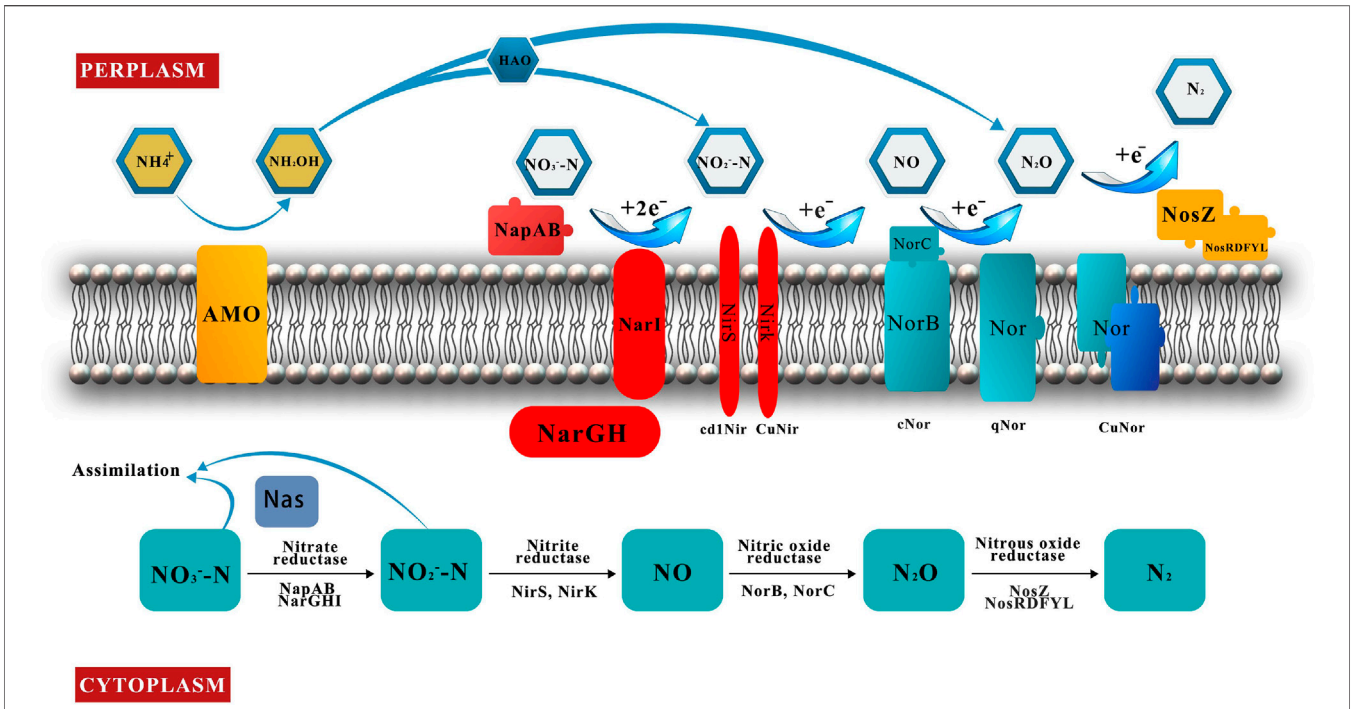

FIGURE 3 | Schematic of nitrification, denitrification, and assimilation reactions under aerobic conditions.

according to the differences in proton and electron transfer centers: cNor, qNor, and Cu_ANor (Hino et al., 2010; Matsumoto et al., 2012).

Two structurally distinct subunits (NorB and NorC) constitute cNor. NorC is a smaller subunit that is on the periplasmic side and has one *c*-type heme as its electron acceptor. NorB is a larger subunit containing three metal catalytic centers, that is, heme *b*, heme b_3, and nonheme Fe_B. A segment of NOR-encoding gene (2,904 bp *nor*B) that can be translated into a 759 amino acid sequence has been amplified successfully in *Alcaligenes faecalis* NR (Huang et al., 2018). qNor contains only one subunit without heme, and Cu_ANor has two subunits and has been reported in *Bacillus* (Suharti and De Vries, 2005).

4.1.4 Nitrous Oxide Reductase (NOS)

NOS reduces nitrous oxide (N_2O) to nitrogen (N_2), with its site of action located in the periplasm (**Figure 3**). Two copper-containing enzymatic catalytic sites, that is, Cu_A and Cu_Z, are located on the same monomer (Cu_A and Cu_Z) (Brown et al., 2000a; Brown et al., 2000b). The intermediate product (N_2O) is a more harmful greenhouse gas than carbon dioxide (CO_2), and emissions of nitrous oxide must be reduced (Lee et al., 2019), but some microorganisms also emit nitrous oxide into the atmosphere as a terminal gaseous nitrogen (Gaimster et al., 2018). Therefore, reducing nitrogen oxide emissions is a very important issue in protecting the atmosphere. NOS is encoded by the *nosZ* gene and the accessory gene *nosRDFYL* (Honisch and Zumft, 2003), and the expression of the *nosZ* gene has been detected in *B. californica* K1 (Fang et al., 2021).

4.2 Critical Enzymes of Nitrification Process

4.2.1 Ammonia Monooxygenase

The conversion of ammonium into hydroxylamine via AMO is the first step in the HNAD nitrification process, and hydroxylamine is an important intermediate metabolite (Hooper et al., 1997). AMO-containing quinone oxidase has two subunits, which are inhibited by light and chelating agents during oxidation, but copper ions can increase the enzyme activity (Moir et al., 1996). The encoding gene of AMO is *amoA*, which was amplified and 397 bp in *S. pararoseus* Y1. Strain Y1 can convert ammonia into hydroxylamine under aerobic conditions (**Table 2**) (Zeng et al., 2020).

4.2.2 Hydroxylamine Oxidoreductase

Oxidation of hydroxylamine to nitrite by HNADs occurs in the periplasm via HAO. The HAO encoding gene has been expressed successfully in *B. cereus* GS-5 (Rout et al., 2017). Recent reports suggest that HAO catalyzes hydroxylamine to produce nitric oxide rather than nitrite as found in previous studies, and nitric oxide is re-oxidized to nitrite (Caranto and Lancaster, 2017). In addition, hydroxylamine has been found to be oxidized to N_2O by P460 (Caranto et al., 2016; White and Lehnert, 2016) under anaerobic conditions and HAO enzymes (Zhao et al., 2019) under aerobic conditions in both ammonia oxidizing bacteria and heterotrophic denitrifying bacteria, respectively.

4.3 Assimilatory Nitrate Reductase (NAS)

Under aerobic conditions, ammonium/nitrate/nitrite assimilation by heterotrophic denitrifying bacteria via NAS is

TABLE 2 | Encoding genes of critical enzymes in nitrogen cycle.

Strain	NR	NIR	Nor	NOS	AMO	HAO	References
Bacillus cereus GS-5	*nar*	*nir*				*hao*	Rout et al. (2017)
Alcaligenes faecalis NR		*nirK*	*norB*	*nosZ*		*hao*	Huang et al. (2018), Zhao et al. (2019)
Pseudomonas stutzeri XL-2	*napA*	*nirS*					Zhao et al. (2018)
Agrobacterium sp. LAD9	*nap*	*nirS*					Chen and Ni (2012)
Sporidiobolus pararoseus Y1	*napA*	*nirK*			*amoA*		Zeng et al. (2020)
Bacillus simplex H-b	*nap*		*nor*				Yang Q. et al. (2021)
Pseudomonas sp. yy7		*nirK*	*norB*	*nosZ*			Wan et al. (2011)
Pseudomonas stutzeri PCN-1		*nirS*	*norB*	*nosZ*			Zheng et al. (2014)
Barnettozyma californica K1		*nirK*		*nosZ*	*amoA*		Fang et al. (2021)

TABLE 3 | Optimal carbon sources for HNADs.

Optimal Carbon Source	Strain	References
Glucose	*Pseudomonas aeruginosa* P-1	Wei et al. (2021)
	Anoxybacillus contaminans HA	Chen et al. (2015)
	Enterobacter cloacae HNR	Guo et al. (2016)
	Sporidiobolus pararoseus Y1	Zeng et al. (2020)
Sodium succinate	*Bacillus methylotrophicus* L7	Zhang et al. (2012)
	Agrobacterium sp. LAD9	Chen and Ni (2012)
	Acinetobacter junii YB	Ren et al. (2014)
	Alcaligenes faecalis SDU20	Chen et al. (2021)
Sodium pyruvate	*Klebsiella* sp. TN-10	Li et al. (2019)
	Pseudomonas chloritidismutans K14	Hou et al. (2021)
Sodium acetate	*Bacillus subtilis* A1	Yang et al. (2011)
	Vibrio diabolicus SF16	Duan et al. (2015)
Sodium citrate	*Acinetobacter* sp. ND7	Xia et al. (2020)
	Acinetobacter sp. T1	Chen et al. (2019)
	Pseudomonas stutzeri GEP-01	Gao et al. (2020)
	Pseudomonas stutzeri YG-24	Li et al. (2015)
Fructose	*Fungus Fusarium solani* RADF-77	Cheng et al. (2020)

an important driving force for nitrogen removal. Nitrate first enters the cell interior through ion channels and then enters the assimilation pathway. Based on the protein sequence and biochemical properties of the catalytic subunits, NAS is classified into three types, that is, NasA, NasB, and NarC (Sparacino-Watkins et al., 2014). *nasABGHC* has been expressed in *Paracoccus denitrificans* PD1222 when nitrate was used as the nitrogen source (Luque-Almagro et al., 2017).

5 ENVIRONMENTAL FACTORS AFFECTING HNADS

5.1 Composition of Culture Medium

The organic carbon source provides nutrients and energy for growth while also being responsible for acting as an electron donor during nitrification and denitrification by HNADs. During the catabolic and anabolic processes of microorganisms, carbon sources provide the necessary nutrients and energy for bacteria (Nancharaiah and Kiran Kumar Reddy, 2018). However, a given HNAD strain exhibits a significantly different growth state, growth rate, and nitrogen-removal rate when using different organic carbon sources (Ren et al., 2014).

5.1.1 Carbon Source

Different carbon sources—such as glucose, acetic acid, succinic acid, citric acid, methanol, and sucrose—have different effects on the growth and nitrogen removal of HNADs, as given in **Table 3**. The molecular weight and chemical structure of the carbon sources vary greatly and have a significant impact on nitrogen removal, and HNAD bacteria usually prefer carboxylates such as acetate to carbohydrates such as glucose (Jia et al., 2019). The reason for this may be that carboxylate has a simple chemical structure and a low molecular weight, making it more conducive to absorption and utilization through the tricarboxylic acid cycle. When performing nitrogen removal under aerobic conditions, sodium acetate is the optimal carbon source for *B. subtilis* A1 and *V. diabolicus* SF16 (Yang et al., 2011; Duan et al., 2015), and sodium pyruvate and succinate are the best recommended carbon sources for *Klebsiella* sp. TN-10 and *B. methylotrophicus* L7 (Zhang et al., 2012; Li et al., 2019). However, other strains such as *P. aeruginosa* P-1, *A. contaminans* HA, and *E. cloacae*

TABLE 4 | Ranges of C/N for HNADs.

Strain	C/N gradient	Optimal C/N	References
Acinetobacter sp. Y16	1, 2, 3, 4, 5, 6, 7, 8	2	Huang et al. (2013)
Acinetobacter sp. ND7	2, 4, 6, 8, 10	8	Xia et al. (2020)
Acinetobacter junii YB	2, 5, 10, 15	10 or 15	Ren et al. (2014)
Acinetobacter sp. T1	3, 5, 10, 15	10	Chen et al. (2019)
Acinetobacter sp. JR1	4, 8, 12, 16, 20, 24	12–24	Yang et al. (2019)
Alcaligenes faecalis SDU20	2, 5, 10, 15, 20	10	Chen et al. (2021)
Fusarium solani RADF-77	2, 5, 10, 15, 40	10	Cheng et al. (2020)
Klebsiella sp. TN-10	2, 4, 8, 12, 16	12	Li et al. (2019)
Marinobacter NNA5	2, 4, 6, 8, 10, 15, 20	6–8	Liu et al. (2016)
Photobacterium sp. NNA4	2, 4, 6, 8, 10, 15, 20	>10	Liu et al. (2019)
Paracoccus denitrificans DYTN-1	5, 10, 15, 20, 25	15	Zhao et al. (2020)
Pseudomonas balearica RAD-17	2, 5, 10, 15, 20	5–15	Ruan et al. (2020)
Pseudomonas aeruginosa P-1	5, 10, 15, 20, 25, 30	15	Wei et al. (2021)
Pseudomonas chloritidismutans K14	2, 6, 10, 14, 18	10	Hou et al. (2021)
Pseudomonas stutzeri SDU10	5, 10, 15, 20	10	Chen et al. (2020)
Pseudomonas stutzeri YG-24	2, 4, 6, 8, 10	8	Li et al. (2015)
Pseudomonas stutzeri UFV5	4, 6, 8, 12	6–10	Silva et al. (2020)
Vibrio diabolicus SF16	4, 6, 8, 10, 12, 14	10	Duan et al. (2015)
Serratia marcescens W5	0, 1, 2, 4, 6, 8, 10, 15, 20, 25, 30, 40	6–20	Wang et al. (2016)

HNR prefer glucose to carboxylate (Wei et al., 2021; Chen et al., 2015; Guo et al., 2016).

As well as conventional carbon sources, some solid ones have been tried in denitrification applications. As a by-product of tea extraction, tea residue has been used as a solid carbon source to cultivate the fungus *F. solani* RADF-77 and can be used as a biofilm attachment carrier in denitrification systems (Cheng et al., 2020). Wood-chip leachate has also been used as a carbon source in the nitrogen removal of *P. tropicum* IS0293 under aerobic conditions (Yao et al., 2020).

5.1.2 Carbon-to-Nitrogen (C/N) Ratio

The C/N ratio affects the growth metabolism, energy conversion, and denitrification efficiency of HNADs, and it is a parameter that reflects the electron donor and acceptor requirements (Huang and Tseng, 2001; Rajta et al., 2020). A low carbon-source concentration affects not only the growth requirements of the strain but also the uptake and conversion efficiency of the microorganisms regarding the nitrogen source, and the optimal C/N ratio for most HNADs is between 8 and 15 (**Table 4**).

In *Acinetobacter* sp. FYF8, it has been reported that the nitrogen-gas conversion ratio is 39.88, 68.85, and 78.79% at a C/N ratio of 2.0, 2.5, and 3.0, respectively (Fan et al., 2021); these results show that the C/N ratio has a significant effect on nitrogen conversion by a given strain under particular incubation conditions. In addition, different nitrogen conversion pathways also require different C/N ratios; for *B. methylotrophicus* L7, the optimal C/N ratio is six for heterotrophic nitrification but 20 for aerobic denitrification (Zhang et al., 2012).

Within certain ranges of carbon-source concentration and C/N ratio, the higher the C/N ratio, the faster the bacterial growth and the higher the nitrogen-removal efficiency (Patureau et al., 2000). It has been found that the denitrification rate of *A. junii* YB increases with increasing C/N ratio in the range of 2–15, and nitrogen-balance analysis has shown that more ammonium is converted to biological nitrogen for cell growth at high C/N ratio (Ren et al., 2014). However, although it has been reported that a higher C/N ratio is more favorable for microbial growth and ammonium removal (Chen and Ni 2012), the latter can be inhibited if the C/N ratio is too high (Wang et al., 2016; Zhao et al., 2020). Most HNADs prefer a high C/N ratio, but some, such as *Acinetobacter* sp. Y16, can have high nitrogen-removal capacity at low C/N ratio (i.e., 2), which is a great advantage when treating wastewater with a low C/N ratio (Huang et al., 2013).

5.1.3 Effect of Inorganic Ions on HNADs

The inorganic ions Cu^{2+}, Mg^{2+}, Ni^{2+}, Pb^{2+}, Mn^{2+}, Zn^{2+}, Fe^{3+}, and Cr^{6+}, along with other heavy metal ions, are ubiquitous in wastewater and are either toxic or potent and affect the nitrogen-removal efficiency of HNADs (He T. et al., 2019). The nitrogen-removal efficiency of *Arthrobacter arilaitensis* Y-10 is significantly higher—by 10.88%—when compared with the control in the presence of copper at 0.1 mg/L but is strongly inhibited in the presence of copper at 0.25 mg/L (He T. et al., 2019). These results show that a low concentration of copper ions helps in ammonia–nitrogen conversion, but a high concentration reduces the removal efficiency.

Metal ions affect not only the growth of the strain but also its ability to remove nitrogen, and the type and concentration of ions have significantly different effects. When metal-ion addition experiments were conducted with *A. aeruginosa* HN-02, a low concentration of Cu^{2+} (0.5 mg/L) barely affected its ammonium-removal efficiency, but it decreased by ~83% at 1.0 mg/L and became almost zero at 1.5 mg/L; compared to Cu^{2+}, the ammonium-removal efficiency with the addition of Zn^{2+} (two to eight mg/L) was essentially the same as that of the control, and the toxicity of Cu^{2+} and Zn^{2+} in co-existence was much higher than that of single-addition Cu^{2+} (Chen et al., 2014). However, certain metal ions—such as Fe^{3+}—can improve the functionality of the microorganism by stimulating its metabolism. For *P. stutzeri* T13, it has been reported that the nitrogen-assimilation capacity and maximum nitrate reduction rate increase significantly upon adding Fe^{3+} (Feng et al., 2021).

TABLE 5 | Ranges of salinity for HNADs.

Strain	Salinity gradient (g/L)	Optimal salinity (g/L)	References
Acinetobacter sp. JR1	0, 5, 10, 15, 20, 25, 30	<15	Chen et al. (2019)
Alcaligenes faecalis SDU20	0, 10, 20, 30, 40, 50	<40	Chen et al. (2021)
Bacillus litoralis N31	5, 10, 20, 30, 40, 50	30–40	Chen et al. (2014)
Bacillus methylotrophicus L7	0, 10, 20, 30, 40	<30	Zhang et al. (2012)
Marinobacter NNA5	0, 10, 20, 30, 40, 60, 80	30–40	Liu et al. (2016)
Pseudomonas aeruginosa P-1	0, 10, 20, 30, 40, 50	<40	Wei et al. (2021)
Pseudomonas stutzeri UFV5	0, 30, 60, 90, 120, 150	<60	Silva et al. (2020)
Pseudomonas balearica RAD-17	0, 2.5, 5.15, 25	<25	Ruan et al. (2020)
Serratia marcescens W5	0, 0, 5, 1.0, 1.5, 2.0, 2.5, 3.5, 5, 9, 15, 20, 25, 30	<25	Wang et al. (2016)

TABLE 6 | Ranges of pH for HNADs.

Strain	pH gradient	Optimal pH	References
Acinetobacter sp. T1	6.5, 7.0, 7.5, 8.0, 8.5	8.5	Chen et al. (2019)
Alcaligenes faecalis SDU20	5, 6, 7, 8, 9, 10	8.0	Chen et al. (2021)
Aeromonas sp. HN-02	2.3, 4, 6, 7, 8.1, 9, 10, 11	8.1	Chen et al. (2014)
Acinetobacter junii YB	5, 6, 7, 7.5, 8, 9, 10	7.5	Ren et al. (2014)
Bacillus methylotrophicus L7	5, 6, 7, 8, 9, 10	7–8	Zhang et al. (2012)
Fusarium solani RADF-77	3.5, 4.97, 6.03, 6.7, 8.3	4.97–6.70	Cheng et al. (2020)
Klebsiella sp. TN-10	4, 5, 6, 7, 8, 9	7	Li et al. (2019)
Marinobacter NNA5	5, 6, 7, 7.5, 8, 9, 10	7.5	Liu et al. (2016)
Photobacterium sp. NNA4	5, 6, 7, 8, 9, 10	7–8	Liu et al. (2019)
Pseudomonas chloritidismutans K14	5.5, 6.5, 7.5, 8.5	7.5	Hou et al. (2021)
Pseudomonas stutzeri SDU10	5, 6, 7, 8, 9, 10	7	Chen et al. (2020)
Serratia marcescens W5	4, 5, 6, 7, 8, 9, 10, 11, 12	7–10	Wang et al. (2016)

Compared with other nitrifying bacteria, *Cupriavidus* sp. S1 shows high nitrogen-removal capacity and excellent resistance to metal ions when various highly concentrated metal ions are added (Sun et al., 2016). In the presence of a high concentration of copper ions, *Pseudomonas aeruginosa* ZN1 has a copper-resistance gene and protein that allow the strain to grow and remove nitrogen at a copper–ion concentration of 800 mg/L (Zhang N. et al., 2018).

5.1.4 Effect of Salinity on HNADs

Salinity affects the denitrification capacity and cell growth of HNADs. High osmotic pressure may lead to strain disintegration, resulting in the loss of either cellular or enzymatic activity; therefore, high concentrations of salt inhibit cell growth and nitrogen removal. Previous studies have shown that most HNADs tolerate salinity below 40 g/L NaCl (**Table 5**), and halotolerant HNADs (Ventosa et al., 1998) may be more advantageous for treating wastewater with high salinity.

Although a low salt concentration is more conducive to cell growth and reproduction, some bacteria isolated from specific environments such as seawater and high-saline effluents have higher nitrogen-removal efficiency with appropriate salinity. In *B. litoralis* N31 isolated from the marine environment, it has been shown that an appropriate salinity (30–40 g/L) is beneficial for removing ammonium, probably because this is more suitable for enzyme activity (Huang F. et al., 2017). Similar phenomena have also been reported for *Marinobacter* NNA5 (Liu et al., 2016).

It has been found that pH and salinity also interact in nitrogen removal. In salinity tests, *S. marcescens* W5 showed a significant difference in ammonium removal between pH seven and pH 10 (Wang et al., 2016). However, studies on the mechanisms that produce this phenomenon are very limited.

5.2 Effects of Culture Conditions on HNADs

5.2.1 Effect of Initial pH

Most HNADs are more adapted to neutral-to-alkaline environments (pH 6.0–9.0), and the optimal pH range for nitrogen removal is 7.0–8.0 (**Table 6**) (Chen et al., 2014; Liu et al., 2016; Hou et al., 2021). Some bacteria such as *Acinetobacter* sp. JR1 and *S. marcescens* W5 have a broad spectrum of pH adaptability (pH 4.5–10.0) (Wang et al., 2016; Yang et al., 2019). For *S. marcescens* W5 and *P. stutzeri* PCN-1, high ammonium-removal efficiency has been reported under both neutral and alkaline conditions (Zheng et al., 2014; Wang et al., 2016). Few strains can grow at extreme pH values, but strain HN-02 shows excellent acid adaptability and can grow cells and remove nitrogen at a pH value as low as 2.3 (Chen et al., 2014). Fungi are more advantageous than bacteria in converting organic nitrogen sources because of their better acid tolerance and denitrification ability (Cheng et al., 2020). HNADs have a broad spectrum of pH adaptability and can be used in various wastewater treatments.

The best growth state and highest nitrogen-removal efficiency of HNADs are usually found in the optimum pH range, such as for *Acinetobacter* sp. T1, *Klebsiella* sp. TN-10, and *Marinobacter* NNA5 (Liu et al., 2016; Chen et al., 2019; Li et al., 2019). However, the optimal cell-proliferation pH and the highest nitrogen-

TABLE 7 | Ranges of temperature for HNADs.

Strain	Temperature gradient (°C)	Optimal temperature (°C)	References
Acinetobacter sp. ND7	25, 30, 35, 40	35	Xia et al. (2020)
Alcaligenes faecalis SDU20	20, 25, 30, 35, 40	30	Chen et al. (2021)
Acinetobacter junii YB	10, 20, 30, 37	37	Ren et al. (2014)
Acinetobacter sp. JR1	10, 20, 30, 37, 40	30	Yang et al. (2019)
Bacillus methylotrophicus L7	20, 25, 30, 37	37	Zhang et al. (2012)
Klebsiella sp. TN-10	10, 20, 30, 37	30	Li et al. (2019)
Marinobacter NNA5	6, 25, 30, 35, 40, 45	35	Liu et al. (2016)
Photobacterium sp. NNA4	16, 23, 30, 37, 45	30–37	Liu et al. (2019)
Pseudomonas balearica RAD-17	5, 15, 25, 40	15–40	Ruan et al. (2020)
Pseudomonas chloritidismutans K14	19, 23, 27, 31, 35	27	Hou et al. (2021)
Pseudomonas stutzeri SDU10	20, 25, 30, 35, 40	30	Chen et al. (2020)
Pseudomonas stutzeri UFV5	20, 25, 30, 35, 40	20–40	Silva et al. (2020)
Serratia marcescens W5	10, 15, 20, 25, 30, 35, 40	15–35	Wang et al. (2016)
Acinetobacter sp. Y16	2–35	20	Huang et al. (2013)

TABLE 8 | Ranges of dissolved oxygen (DO) for HNADs.

Strain	DO gradient (rpm)	Optimal DO (rpm)	References
Acinetobacter sp. T1	40, 80, 120, 160, 200	160	Chen et al. (2019)
Acinetobacter junii YB	80, 120, 160, 200	160	Ren et al. (2014)
Acinetobacter sp. JR1	0, 40, 80, 120, 160, 200	120	Yang et al. (2019)
Fusarium solani RADF-77	100, 150, 200	150–200	Cheng et al. (2020)
Marinobacter NNA5	50, 100, 150, 200	150	Liu et al. (2016)
Photobacterium sp. NNA4	50, 100, 160, 200	160	Liu et al. (2019)
Pseudomonas chloritidismutans K14	20, 60, 100, 140, 180	140	Hou et al. (2021)

removal pH of *Photobacterium* sp. NNA4 are inconsistent, at 5.0–6.0 and 7.0, respectively (Liu et al., 2019).

5.2.2 Effect of Temperature

Temperature affects microbial growth and the catalytic efficiency of enzymatic activity, and the optimal temperature range for HNADs is 30–37°C (**Table 7**) (Li et al., 2019; Liu et al., 2019; Xia et al., 2020). The higher the temperature in the enzyme-activity temperature range, the higher the enzyme reaction rate; the nitrification efficiency doubles for each 10 °C increase, and the denitrification efficiency doubles for each 4 °C increase (Zaitsev et al., 2008; Wu et al., 2019). The growth of most HNADs is inhibited at low temperature, but at 2 °C, *Acinetobacter* sp. Y16 can still achieve half of its nitrogen-removal efficiency at the optimal temperature (20 °C), with good low-temperature adaptability (Huang et al., 2013). The optimal temperature for *B. simplex* H-b is 10 °C, which is lower than that for other HNADs (Yang Q. et al., 2021). The growth trend of microorganisms is usually consistent with nitrogen removal, but at lower temperatures, microbial growth requires a longer lag period. Low-temperature nitrogen-removal HNADs can be used in wastewater treatment in cold areas.

5.2.3 Dissolved Oxygen

In contrast to denitrifying bacteria, DO affects HNAD microbial colonization and nitrogen-removal efficiency and is a control parameter of the denitrification pathway (Hocaoglu et al., 2011). Conventional anaerobic denitrifying bacteria use nitrate as an electron acceptor to obtain energy, but the aerobic HNADs require the participation of oxygen to act as an electron acceptor for the denitrification process (Yang J. et al., 2020). In nitrogen removal by the denitrification pathway, nitrite reductase is sensitive to DO and can be inhibited by high DO concentration (Ka et al., 1997). It has been found that nitrite removal by *P. stutzeri* T13 is increased significantly by reducing the DO concentration and adjusting the rotational speed: Low rotational speed with low DO concentration is more useful for removing nitrite (Sun et al., 2015). HNADs have adapted to high-DO conditions for nitrogen removal and have higher efficiency than anaerobic denitrifying bacteria, with the optimal speeds given in **Table 8**.

The DO level is usually achieved by adjusting the rotation rate, but the DO concentration differs under different experimental conditions. The optimal shaking speed for both *Acinetobacter* sp. T1 and *Photobacterium* sp. NNA4 is 160 rpm, but their respective DO values are 5.1 mg/L and 5.89 mg/L (Chen et al., 2019; Liu et al., 2019).

Acinetobacter sp. ND7 requires different levels of DO for ammonium removal via the nitrification pathway and for nitrate removal via the denitrification pathway, with high DO favoring ammonium removal and low DO favoring nitrate removal (Xia et al., 2020).

5.3 Effect of Antibiotics on HNADs

With the widespread use of antibiotics in aquaculture, agriculture, and medicine worldwide, antibiotic residues affect the removal of nitrogen. Sulfamethoxazole (>2 μg/L) damages denitrifying cells,

reduce bacterial electron-transport activity, and inhibit N_2O reduction, leading to a reduction in aerobic denitrification nitrogen-removal efficiency in *P. stutzeri* PCN-1 (Gui et al., 2017). However, the nitrogen-removal efficiency of *P. balearica* RAD-17 through the aerobic denitrification pathway is insensitive to low and high concentrations of antibiotics such as ciprofloxacin and hygromycin (Ruan et al., 2021).

5.4 Effect of Nanoparticles on HNADs

In the past decade, ZnO and CuO nanoparticles (NPs) have been used widely in manufacturing industries and have diffused into the environment, posing a potential risk to the nitrogen-removal process (Huang et al., 2010). In *P. aeruginosa* PCN-1, ZnO NPs inhibit the gene expression of nitrate and nitrite reductases, thereby affecting the catalytic activity of the enzymes and leading to a significant accumulation of nitrite (Chen et al., 2017). Uncoincidentally, the presence of ZnO NPs inhibits NR and NIR activities, reduces the denitrification efficiency of *Clostridium perfringens* HNR, and leads to increased production of extracellular polymeric substances (Ma et al., 2020). In addition, in *P. tolaasii* Y-11, CuO NPs have been found to not only affect nitrate reduction through their own action but also cause Cu^{2+}-inhibited ammonium conversion (Yang Y. et al., 2020).

By contrast, the growth and nitrogen-removal capacities of *P. tolaasii* Y-11 are increased significantly in the presence of Fe^{2+} because of the polymerization of ZnO and CuO NPs promoted by Fe^{2+} (Yang Y. et al., 2020; Yang Y. et al., 2021). Also, the presence of phosphate reduces significantly the concentration of toxic Zn(II) released from ZnO NPs in the medium, and it weakens the effect of ZnO NPs on nitrogen removal (Cheng et al., 2019).

6 USE OF HNADS IN SEWAGE TREATMENT

The use of HNADs in practical wastewater treatment has been investigated. *P. denitrificans* Z195 was inoculated into raw wastewater obtained from wastewater treatment plants, and the bacteria had excellent total nitrogen removal and denitrification performance (Zhang et al., 2020). Single HNAD strains have been studied widely, but mixed cultures of aerobic denitrifying bacteria have attracted less attention. A30, D10, and Z40 are three new bacteria isolated from sediment and inoculated into actual wastewater; 86% of total nitrogen and 93% of chemical oxygen demand (COD) were removed (Zhang H. et al., 2019). Huang F. et al. (2020) reported that a consortium of four novel bacteria (*Marinomonas*, *Marinobacterium*, *Halomonas*, and *Cobetia*) could convert ammonium into biological (~60%) and gaseous (36–38%) nitrogen, with significantly lower denitrification by a single strain alone than by the mixture.

The use of HNADs in agricultural and industrial wastewater treatment has also been investigated. In swine wastewater treatment, the highest removal of ammonium and COD by *P. stutzeri* SDU10 can reach 97.6 and 94.2%, respectively, with the presence of sodium acetate to adjust the C/N ratio to 10 (Chen et al., 2020). Similar results have been reported for *Acinetobacter* sp. T1, which can significantly improve the nitrogen removal of pig-farm wastewater when compared with traditional activated sludge (Chen et al., 2019). In addition, S. *marcescens* CL1502 isolated from North Atlantic deep-sea sediments has shown efficient conversion of total nitrogen, ammonium, and COD from actual tannery-industry wastewater (Huang G. et al., 2017).

The use of feed has promoted the rapid development of aquaculture, but it also brings significant environmental problems, especially nitrogen pollution. The presence of *P. stutzeri* SC221-M in an experimental aquaculture system reduced pollutant production, improved water quality, and influenced the microbial community structure (Deng et al., 2014).

7 CONCLUSION AND OUTLOOK

HNADs are isolated from soil, sludge, and sewage; they are mostly bacteria, but a few are fungi. Nitrogen is not only assimilated into organic nitrogen required for cell growth but also converted into gaseous nitrogen by HNADs under aerobic conditions. Ammonium conversion is achieved through three different metabolic pathways, and hydroxylamine is an important intermediary therein. The critical enzymes and encoding genes of the nitrification and denitrification metabolic pathways are also studied, as is optimizing the carbon source, C/N, metal ions, salinity, pH, temperature, and DO to improve the nitrogen-removal efficiency. Fe^{2+} and phosphoric acid reduce the effect of NPs on nitrogen removal. HNAD bacteria have excellent application potential in treating breeding and leather wastewater, removing nitrogen sources, and improving water quality.

Currently, most research into HNADs is focused on isolation and strain identification. Research into metabolic transformation mechanisms and the transformation of key enzyme encoding genes is still very limited, and the use of HNADs in sewage treatment needs to be improved.

AUTHOR CONTRIBUTIONS

WF: investigation, visualization, writing—review and editing; GS: project administration and supervision; YW: investigation and visualization; QW: writing—review and editing; PD: investigation; CL: investigation; XZ: conceptualization and project administration; ZR: conceptualization, supervision, and funding acquisition.

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Conflict of Interest: GS, YW, and PD were employed by Fujian Dabeinong Huayou Aquatic Science and Technology Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.