

Effects of Bacillus Characteristics on Nitrogen Transformation in Aquaculture Water

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Date: 2022-05-17T00:00:00+00:00

Abstract

[Objective] To investigate the effects of strain characteristics and water environment on nitrogen transformation by *Bacillus* under aquaculture conditions. [Methods] The hydrolytic enzyme activities, antibacterial activities, and growth capabilities of two *Bacillus* strains NT9 and YB3 were determined, and path analysis was performed on nitrogen transformation during simulated aquaculture processes. [Results] The results showed that strain NT9 belongs to *Bacillus subtilis* and is capable of ammonium nitrogen transformation even under low-nutrient conditions, with a removal rate of 89.3%. Compared with strain YB3, it exhibited stronger protease and amylase activities as well as antibacterial activity, but poorer growth capability. In simulated aquaculture water, both strains had little effect on water quality parameters such as nitrate nitrogen, nitrite nitrogen, dissolved oxygen, pH, and alkalinity, and could promote organic nitrogen degradation, but showed poor ammonium nitrogen transformation efficiency, leading to significant accumulation of ammonium nitrogen in the water ($P < 0.05$). Path analysis revealed that total bacterial count in water (cfu) was the main factor promoting organic nitrogen degradation, with a path coefficient (direct effect) of 0.550 ($P < 0.01$). The cellulase activity (cel) and growth capability (gro) of the strains exerted substantial indirect effects on organic nitrogen degradation through cfu. Meanwhile, microbial growth and proliferation activities (total bacterial increment, dcfu) were the primary factors promoting ammonium nitrogen transformation, with a path coefficient of -0.112 ($P < 0.01$). [Conclusion] The study demonstrated that both *Bacillus* strains could promote feed protein degradation under aquaculture conditions, but the ammonium nitrogen transformation effect was not significant. According to the path analysis results, research on synchronous ammonium nitrogen transformation could be conducted by promoting *Bacillus* growth.

Full Text

Effect of Bacillus Properties on Nitrogen Conversion in Aquaculture Water Bodies

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[Objective] This study investigated the effects of strain characteristics and water environmental conditions on nitrogen conversion by *Bacillus* in aquaculture settings. **[Methods]** We determined the hydrolytic enzyme activities, bacteriostatic activities, and growth capabilities of two *Bacillus* strains (NT9 and YB3) under oligotrophic conditions, and subsequently analyzed their nitrogen conversion effects in a simulated aquaculture experiment using path analysis. **[Results]** Strain NT9 was identified as *Bacillus subtilis*, which exhibited ammonia nitrogen conversion functionality even under low-nutrient conditions, achieving a removal rate of 89.3%. Compared with strain YB3, NT9 demonstrated stronger protease and amylase activities as well as greater bacteriostatic activity, but exhibited poorer growth capability. In simulated aquaculture water bodies, both strains had minimal impact on water quality parameters including nitrate nitrogen, nitrite nitrogen, dissolved oxygen, pH, and alkalinity. While both strains promoted organic nitrogen degradation, their ammonia nitrogen conversion efficiency was poor, resulting in significant ammonia accumulation ($P < 0.05$). Path analysis revealed that total bacterial count (cfu) in the water was the primary factor promoting organic nitrogen degradation, with a path coefficient (direct effect) of 0.550 ($P < 0.01$). Cellulase activity (cel) and growth ability (gro) exerted substantial indirect effects on organic nitrogen degradation through cfu. Meanwhile, microbial growth and proliferation (total bacterial increment, dcfu) represented the main factor promoting ammonia nitrogen conversion, with a path coefficient of -0.112 ($P < 0.01$). **[Conclusions]** Both *Bacillus* strains enhanced feed protein degradation under aquaculture conditions, but showed limited ammonia nitrogen conversion efficiency. Based on path analysis results, future research should focus on promoting *Bacillus* growth to achieve synchronous ammonia nitrogen conversion.

Keywords: *Bacillus*; Aquaculture water body; Nitrogen conversion; Influencing factors; Path analysis

In aquaculture, only 20-30% of feed protein nitrogen is ultimately absorbed and utilized by aquatic animals, with the remainder being released into the water environment [1-2]. It is estimated that 78% of nitrogen in aquaculture water originates from feed [3-4], primarily existing in organic form [5]. This nitrogen enrichment causes pollution, while the degradation of organic nitrogen into inorganic forms such as ammonia, nitrite, and nitrate negatively impacts aquatic animal health [6]. *Bacillus* species possess multiple functional activities, including secretion of proteases, amylases, and cellulases, which can accelerate nitrogen transformation in aquaculture water, reduce eutrophication risks, and minimize accumulation of ammonia and nitrite [7-9], making them highly valuable for addressing these challenges [10].

However, the nitrogen conversion efficiency of *Bacillus* is influenced by numerous factors, including cultivation conditions such as temperature, pH, dissolved oxygen, nitrogen source, and carbon source [11-14], as well as intrinsic strain characteristics like growth capability, hydrolytic enzyme activity, and bacteriostatic activity [15]. Particularly under aquaculture conditions, where nutrient levels are substantially lower than in microbial culture media (total nitrogen 29 mg · L⁻¹ vs. 1000 mg · L⁻¹) [16], *Bacillus* may form spores and enter dormancy due to nutrient deficiency, ultimately affecting nitrogen conversion efficiency [17]. Consequently, the factors influencing *Bacillus* nitrogen conversion in aquaculture water are more complex and difficult to quantitatively compare through single-factor experiments [11-13].

Path analysis is a multivariate statistical technique that, unlike other methods, can decompose the total effect of independent variables into standardized direct and indirect effects through effect partitioning. This allows for intuitive comparison of various factors' contributions and provides reliable identification of primary factors, making it suitable for comprehensive multi-factor analysis. The technique has been widely applied in aquaculture breeding and production [18-20], though its application in *Bacillus* nitrogen conversion research remains unreported.

Therefore, this study employed path analysis to investigate the effects of strain characteristics, water quality conditions, and other factors on nitrogen conversion by two *Bacillus* strains (NT9 and YB3) in simulated aquaculture processes. The objective was to identify key factors influencing *Bacillus* nitrogen conversion efficiency and explore potential approaches for enhancing nitrogen transformation, thereby providing a foundation for efficient *Bacillus* application in aquaculture water.

1.1 Bacterial Strains

Strains NT9 and YB3 were preserved in the Key Laboratory of Health Aquaculture and Product Processing in Dongting Lake Area, Hunan Province. Strain NT9 was isolated from traditional fermented food (Labadou), while strain YB3, identified as *Bacillus cereus*, was isolated from aquaculture water and possesses

organic nitrogen degradation and ammonia nitrogen removal capabilities [21]. Pathogenic bacteria including *Edwardsiella* sp. XCP, *Aeromonas hydrophila* HTS, *Pseudomonas fluorescens* TLF, *Vibrio alginolyticus* HTL, *Escherichia coli*, and *Staphylococcus aureus* were preserved in the Zoology Key Laboratory of Hunan Higher Education.

1.2 Culture Media

LB medium contained: soybean peptone (8.0% total nitrogen) 10.0 g, yeast extract (10.0% total nitrogen) 5.0 g, glucose 10.0 g, NaCl 5.0 g, water 1000 mL, pH 7.0. For solid medium, 12.0 g agar was added. Cellulase detection medium contained: sodium carboxymethyl cellulose 5.0 g, soybean peptone 10.0 g, NaCl 5.0 g, KH_2PO_4 0.5 g, agar 12.0 g, water 1000 mL, pH 7.0. Casein agar and starch media were prepared according to Li [22] for protease and amylase activity detection, respectively.

1.3 Strain Identification

Strain NT9 was identified through morphological characterization, 16S rRNA gene cloning, sequencing, and phylogenetic analysis following the methods described by Huang et al. [21].

1.4 Growth and Ammonia Nitrogen Conversion Under Low-Nutrient Conditions

Following Huang et al. [21] with minor modifications, we investigated growth and ammonia conversion using NH_4Cl as the sole nitrogen source at 0.01% (v/v) concentration with 0.1% (v/v) glucose as carbon source. Inoculum was prepared to achieve an initial OD_{600} of 0.010, followed by incubation at 30°C with shaking at 150 $\text{r} \cdot \text{min}^{-1}$. Cultures were centrifuged at 12,000 $\text{r} \cdot \text{min}^{-1}$ for 20 min at 4°C, washed three times with 0.6% sterile saline, resuspended in equal volume, and OD_{600} was measured. Ammonia, nitrite, and nitrate concentrations in the supernatant were determined [23]. Parameters were calculated as follows:

Ammonia conversion rate (R , %) = “!#”

Ammonia conversion speed (S , $\text{mg}/(\text{L} \cdot \text{d})$) = “!#” “#!

where $c\%$ represents the maximum ammonia concentration (mg/L), $c\&$ is the final ammonia concentration (mg/L), $t\%$ is the time (d) when ammonia concentration peaked, and $t\&$ is the experimental end time (d).

1.5 Strain Activity Assays

Protease, amylase, and cellulase activities were determined according to Li [22], with relative activity calculated as:

Hydrolase relative activity (H , %) = $\times 100$

where s represents hydrolysis zone area (mm^2) and s) represents colony area (mm^2).

For bacteriostatic activity assessment, *Bacillus* and six pathogenic strains were cultured in LB broth at 30°C and 150 r/min for 24 h, centrifuged at 12,000 r/min for 10 min at 4°C, and resuspended in 0.6% sterile saline to prepare suspensions with $OD_{600} = 0.1$. Pathogen suspensions (100 L each) were spread on LB agar plates, allowed to stand for 30 min at room temperature, then spotted with 50 L *Bacillus* suspension. After 30 min, plates were co-cultured at 30°C for 48 h. Colony and inhibition zone diameters were measured to calculate relative bacteriostatic activity:

$$\text{Relative bacterial inhibition activity (I, \%)} = \frac{s^*}{s} \times 100$$

where s^* represents inhibition zone area (mm^2) and s represents colony area (mm^2).

To compare growth capability under low-nutrient conditions, *Bacillus* suspensions were inoculated into 1:99 diluted LB broth to achieve initial $OD_{600} = 0.010$, incubated at 30°C for 96 h, with OD_{600} measured continuously in triplicate.

1.6 Simulated Aquaculture Experiment

The simulated aquaculture experiment followed Huang et al. (2019) with modifications.

(1) Simulated Aquaculture Water Preparation

Twelve 200 L HDPE plastic tanks were filled with 100 L tap water (pH 7.20). pH was adjusted to approximately 8.00 using sodium bicarbonate (analytical grade, Sinopharm Chemical Reagent Co., Ltd.), disinfected with $10 \text{ mg} \cdot \text{L}^{-1}$ chlorine dioxide for 24 h, then neutralized with vitamin C ($1 \text{ mg} \cdot \text{L}^{-1}$) to eliminate residual toxicity (Lara et al., 2017; Prata Gaona et al., 2017).

(2) Inoculum Preparation and Water Inoculation

Strains were cultured in LB broth at 30°C and $150 \text{ r} \cdot \text{min}^{-1}$ for 24 h to prepare seed cultures. Seed cultures were transferred to fresh LB broth at 1:100 ratio, incubated under the same conditions for 24 h, then centrifuged at $12,000 \text{ r} \cdot \text{min}^{-1}$ for 10 min at 4°C. Pellets were washed and resuspended in 0.6% sterile saline before addition to six simulated aquaculture tanks to achieve initial total bacterial counts of $1.0 \times 10^6 \text{ cfu} \cdot \text{mL}^{-1}$. Tanks inoculated with strains NT9 and YB3 were designated NT9 and YB3 water bodies, respectively.

(3) Simulated Aquaculture

Commercial *Litopenaeus vannamei* feed (crude protein \$40.0%, crude fat \$5.0%, crude fiber \$5.0%, crude ash \$16.0%, moisture \$12.0%) for the first 3 days and $1.0 \text{ g} \cdot \text{d}^{-1}$ thereafter, with no feeding on days 8 and 20. The 24-day experiment involved no water exchange with continuous aeration.

(4) Water Quality Measurement

Dissolved oxygen, temperature, and pH were measured twice daily at 08:00 and 18:00 using a YSI-550A multiparameter water quality analyzer (YSI, USA). Other parameters were measured every 4 days. Ammonia, nitrite, nitrate, carbonate alkalinity, and calcium-magnesium hardness were determined according

to standard methods [23]. Total bacterial counts were measured every 4 days using plate counting methods.

1.7 Data Analysis

Except for total bacterial counts (single measurement per sample), all data are presented as mean \pm standard deviation (SD). Normality and homogeneity of variance were tested using SPSS 16.0. Student's *t*-test was used to compare hydrolase activities, bacteriostatic activities, and growth capabilities between strains. One-way ANOVA was applied to water quality data from simulated aquaculture experiments, with Tukey's multiple comparisons when significant differences were detected ($P < 0.05$). Percentage data were arcsine-transformed, and total bacterial counts were \log_{10} -transformed before analysis. Non-parametric Kruskal-Wallis test was used for non-homogeneous data. Path analysis followed Du and Chen [24], using SPSS 16.0 Regression with Enter method for multiple regression analysis followed by effect partitioning.

2.1 Colony Characteristics and Identification of Strain NT9

Strain NT9 was Gram-positive and spore-forming (Fig. 1a). On LB medium at 30°C for 48 h, colonies were circular (2-5 mm diameter), raised, creamy, viscous, smooth, with regular edges, gray-white, semi-transparent, and often contained turbid opaque flocculent bands internally (Fig. 1b). BLAST analysis revealed its 16S rRNA gene sequence (GenBank Accession No. KX891556) shared 100% similarity with *Bacillus subtilis* subsp. *natto* strains CGMCC 2108 (CP014471) and BEST195 (AP011541). Phylogenetic analysis also clustered this strain with *B. subtilis* (Fig. 1c).

[Figure 1: see original paper]

2.2 Growth and Ammonia Nitrogen Conversion of Strain NT9 Under Low Nitrogen Concentration

As shown in Fig. 2, in low-concentration NH_4Cl medium, strain NT9's OD_{600} decreased within 1 day post-inoculation, then increased to a peak of 0.037 ± 0.006 at day 6, with an average growth rate of 0.0062 absorbance units per day. Ammonia concentration continuously decreased from 27.66 ± 2.43 mg/L to 2.96 ± 0.03 mg/L at day 30, achieving an ammonia conversion rate of 89.3% and conversion speed of 0.82 mg/(L · d). No nitrite or nitrate production was detected.

[Figure 2: see original paper]

2.3 Biological Activities and Growth Characteristics of Strains NT9 and YB3

The protease, amylase, and cellulase activities of strains NT9 and YB3 are shown in Table 1. Strain NT9 exhibited strong protease and amylase secretion, with significantly higher relative activities than strain YB3 ($P < 0.05$), while YB3 showed stronger cellulase activity.

Table 1 Relative hydrolase activities of strain NT9 and YB3

Hydrolases Strain	Hydrolytic zone diameter (mm)	Colony diameter (mm)	Relative activity (%)	
Protease	NT9	21.0 ± 0.5 a	9.6 ± 0.3 a	4.80 ± 0.5 b
	YB3	19.4 ± 0.7 a	13.2 ± 1.0 b	2.20 ± 0.25 a
Amylase	NT9	17.5 ± 0.5 b	8.6 ± 0.5 a	4.15 ± 0.67 b
	YB3	12.8 ± 1.3 a	9.4 ± 0.3 a	1.86 ± 0.26 a
Cellulase	NT9	19.2 ± 1.0	7.5 ± 0.2 a	6.83 ± 1.17
	YB3	§§	7.4 ± 0.2 a	nc

Note: §§ indicates unclear hydrolytic zone; nc, not calculated.

Strain NT9 showed pronounced inhibition against *E. coli*, *S. aureus*, *Edwardsiella* sp. XCP, and *A. hydrophila* HTS, weak inhibition against *P. fluorescens* TLF, and no inhibition against *V. alginolyticus* HTL. Strain YB3 exhibited generally weaker bacteriostatic activity, with only slight inhibition against TLF (Table 2).

Table 2 Relative bacterial inhibition activities of strain NT9 and YB3

Pathogens Strain	Colony diameter (mm)	Inhibition zone diameter (mm)	Relative activity	
<i>E. coli</i>	NT9	8.2 ± 0.8 a	10.1 ± 1.1	1.54 ± 0.02
	YB3	7.7 ± 0.7 a	13.4 ± 0.7	3.82 ± 0.01
<i>P. fluorescens</i> TLF	NT9	6.3 ± 0.9 a	9.8 ± 0.9	1.79 ± 0.01
	YB3	6.9 ± 1.0 a	20.3 ± 0.3	6.96 ± 0.42
<i>Edwardsiella</i> sp. XCP	NT9	6.9 ± 0.3 a	-	-
	YB3	8.4 ± 0.5 a	-	-
<i>V. alginolyticus</i> HTL	NT9	7.3 ± 1.3 a	-	-
	YB3	7.5 ± 0.3 a	-	-
<i>A. hydrophila</i> HTS	NT9	7.3 ± 0.7 a	-	-
	YB3	7.6 ± 0.5 a	-	-
<i>S. aureus</i>	NT9	7.7 ± 0.1 a	-	-
	YB3	8.3 ± 1.0 a	-	-

Pathogens Strain	Colony diameter (mm)	Inhibition zone diameter (mm)	Relative activity
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Fig. 3 shows growth of strains NT9 and YB3 in 1:99 diluted LB broth. Both strains experienced a lag phase (12 h and 24 h, respectively) before entering logarithmic growth, reaching peak OD₆₀₀ at 48 h. Strain NT9 peaked at 0.033 ± 0.013 (growth rate: 0.0008 absorbance units/h), while YB3 peaked at 0.110 ± 0.002 (growth rate: 0.0041 absorbance units/h). Subsequently, YB3's OD₆₀₀ declined rapidly while NT9 remained stable. Overall, YB3's OD₆₀₀ was significantly higher than NT9's during 48-96 h ($P < 0.05$).

[Figure 3: see original paper]

2.4 Effects of Strains on Simulated Aquaculture Water Quality

Table 3 shows that ammonia concentration in YB3 water was significantly higher than in NT9 water and control water ($P < 0.05$), while NT9 water was significantly higher than control ($P < 0.05$). Average total bacterial count in YB3 water was significantly higher than control ($P < 0.05$), but no significant difference existed between NT9 and control waters ($P > 0.05$). No significant differences were observed among the three water types for nitrite, nitrate, temperature, salinity, alkalinity, or hardness ($P > 0.05$). Dissolved oxygen and pH in NT9 and YB3 waters were significantly lower than control ($P < 0.05$), though differences were small.

Table 3 Water quality indices of three different water bodies

Water quality indices	Control	NT9 water	YB3 water
Ammonia concentration (mg/L)	0.04 ± 0.02	8.17 ± 2.33 b	3.46 ± 2.39 a
Total bacterial counts§ ($\times 10^5$ cfu/mL)	0.43 ± 0.13	5.63 ± 0.16 b	1.91 ± 1.95 a
Nitrite concentration (mg/L)	0.27 ± 0.33	5.34 ± 0.20 a	1.91 ± 1.95 a
Nitrate concentration (mg/L)	0.02 ± 0.03	5.34 ± 0.12 a	5.20 ± 1.86 a
Temperature (°C)	27.4 ± 1.1	27.6 ± 1.1	27.5 ± 1.1
Dissolved oxygen (mg/L)	8.13 ± 0.09	8.00 ± 0.11 a	8.05 ± 0.09 a
Salinity (‰)	17.81 ± 0.35	17.94 ± 0.45	18.22 ± 0.59
Alkalinity (mg/L CaCO ₃)	128.3 ± 18.9	122.8 ± 17.1	136.4 ± 29.7
Hardness (mg/L CaCO ₃)	143.2 ± 15.0	137.7 ± 14.8	149.5 ± 28.3

Note: § indicates Kruskal-Wallis test of data after logarithmic transformation.

2.5 Factors Influencing Nitrogen Conversion in Water

In all simulated aquaculture water bodies, nitrogen conversion primarily manifested as ammonia nitrogen accumulation (Table 3). Therefore, path analysis (multiple regression and effect partitioning) was performed with ammonia nitrogen concentration (tan) as the dependent variable and 22 parameters as independent variables, including: bacteriostatic activities (against *E. coli*, *Edwardsiella* sp., *A. hydrophila*, *S. aureus*), hydrolytic enzyme activities (protease, amylase, cellulase), growth ability, inoculation amount, experimental stage, initial total bacterial count, total bacterial increment, ammonia nitrogen increment, total nitrogen content, nitrite and nitrate concentrations, temperature, dissolved oxygen, pH, salinity, alkalinity, and hardness.

Regression analysis showed the model was highly significant ($R^2 = 0.988$, $P < 0.001$), with seven variables included: amylase activity (amy), cellulase activity (cel), growth ability (gro), initial total bacterial count (cfu), experimental stage (time), total bacterial increment (dcfu), and ammonia nitrogen increment (dtan) (Table 4). Direct effects (standardized regression coefficients/path coefficients) of amy, cel, and gro were not significant ($P > 0.05$) but exhibited collinearity [25] with substantial indirect effects (Table 6). cfu, time, and dtan showed highly significant positive direct effects ($P < 0.01$), while cel, gro, dcfu, and dtan exerted large indirect effects through cfu. dcfu had a significant negative direct effect ($P < 0.01$), and cfu, dcfu, and dtan showed negative indirect effects through time (Tables 4 and 6).

Table 4 Multiple regression analysis for nitrogen conversion

Variables	Regression coefficients	Standardized coefficients	Significance	Collinearity VIF
Constant	-0.316*	0.325*	< 0.001	-
amy	0.327*	0.897**	< 0.001	-
cel	0.550**	0.990**	< 0.001	-
gro	-0.112**	0.161**	< 0.001	-
cfu	-	-	-	-
time	-	-	-	-
dcfu	-	-	-	-
dtan	-	-	-	-

Note: amy, amylase activity; cel, cellulase activity; gro, growth ability; cfu, initial total bacterial count; time, experimental stage; dcfu, increment of total bacterial count; dtan, increment of total ammonia nitrogen.

Table 5 Correlations among variables

[Table content showing correlation coefficients between variables]

Table 6 Path analysis and effects division for nitrogen conversion

[Table content showing total effects, direct effects, and indirect effects]

3.1 Comparison of Strain NT9 and YB3 Activities

Bacillus species possess multiple physiological functions, including secretion of proteases, amylases, lipases, and cellulases, which can enhance digestive function in aquaculture animals, promote nutrient absorption, degrade organic matter, accelerate residual feed decomposition, reduce eutrophic element accumulation, and promote ecological cycling in aquatic environments [10]. Strain NT9 was identified as *Bacillus subtilis* (Fig. 1) with strong protease and amylase activities and bacteriostatic properties (Tables 1 and 2). Strain YB3, identified as *Bacillus cereus* [21], showed weaker protease and amylase activities but stronger cellulase activity (Table 1). Original habitat significantly influences strain characteristics, as different ecological environments support distinct dominant species [26]. Strain NT9 was isolated from Labadou, a fermented food rich in soybean protein and starch that favors strains with strong protease and amylase activities [27-28]. In contrast, YB3 originated from low-nutrient aquaculture water where enzyme induction may be weaker. This may explain NT9' s faster environmental adaptation but poorer low-nutrient adaptation. In 1:99 diluted LB broth, NT9' s strong hydrolytic activities (Table 2) provided nutritional advantages, resulting in a shorter lag phase but poorer overall growth (Fig. 3). YB3' s rapid OD₆₀₀ decline after 48-96 h (Fig. 3) likely resulted from nutrient depletion due to earlier rapid growth. Similar results were observed in low-concentration NH₄Cl medium, where NT9' s peak OD₆₀₀ (0.037, Fig. 2) was substantially lower than YB3' s (0.100) [21]. Ammonia concentration continuously decreased without nitrite or nitrate production (Fig. 2), indicating ammonia transformation through assimilation rather than nitrification [29], consistent with YB3' s behavior [21].

3.2 Organic Nitrogen Degradation and Influencing Factors Under Aquaculture Conditions

During the simulated aquaculture process, temperature, dissolved oxygen, pH, salinity, nitrite, nitrate, alkalinity, and hardness in all water bodies remained within suitable ranges for *L. vannamei* [30]. However, all three water types accumulated high ammonia concentrations. NT9 water showed significantly higher average ammonia than control ($P < 0.05$, Table 3), indicating enhanced degradation of feed protein and other organic nitrogen sources. Organic nitrogen degradation relates to microbial enzyme secretion [15], and NT9 possessed strong protease and amylase activities (Table 1). Additionally, *Bacillus* organic nitrogen degradation capability correlates with microbial growth activity, with better growth yielding higher efficiency [33]. YB3 water accumulated significantly higher ammonia than NT9 water ($P < 0.05$, Table 3), suggesting more intense organic nitrogen degradation activity despite YB3' s lower hydrolytic enzyme activities (Table 1). Similar findings by Li [22] and Yan et al. [32] showed that *Bacillus* strains with highest organic nitrogen degradation rates did not nec-

essarily have highest enzyme activities. Path analysis also indicated protease activity had minimal impact on ammonia and was excluded from the model, while amylase and cellulase activities showed non-significant direct effects ($P > 0.05$, Tables 4 and 6). In contrast, total bacterial count (cfu) exhibited highly significant positive direct effects on ammonia concentration ($P < 0.01$, Table 6). In this low-nutrient simulated aquaculture system, YB3' s stronger adaptability (Fig. 3) resulted in more intense organic nitrogen degradation and higher ammonia accumulation (Table 3). Total bacterial increment (dcfu) also promoted organic nitrogen degradation through cfu (0.252, Table 6), consistent with Huang et al. [17] who proposed that microbial quantity effects can be partitioned into base and increment effects. Experimental stage (time) and ammonia nitrogen increment (dtan) also showed highly significant direct effects ($P < 0.01$, Table 6), indicating weak ammonia conversion as both represent cumulative effects. Cellulase activity (cel) and growth ability (gro) benefited organic nitrogen degradation through large indirect effects via cfu (Table 6), likely due to their highly significant positive correlations with cfu ($P < 0.01$, Table 5). Additionally, bacteriostatic activity showed negative correlations with cfu ($r = -0.899$ to -0.901 , $P < 0.001$, data not shown), suggesting NT9' s bacteriostatic properties (Table 2) may inhibit growth of other organic-degrading bacteria such as Proteobacteria and Bacteroidetes [34-35], thereby affecting organic nitrogen degradation.

3.3 Factors Influencing Bacillus Ammonia Nitrogen Conversion Under Aquaculture Conditions

Both strain NT9 (Fig. 2) and YB3 [21] demonstrated ammonia nitrogen conversion capability, yet simulated aquaculture water showed poor conversion efficiency with substantial ammonia accumulation (5.20 - $11.69 \text{ mg} \cdot \text{L}^{-1}$, Table 3), far exceeding the safe concentration for *L. vannamei* ($2.44 \text{ mg} \cdot \text{L}^{-1}$) [6]. Path analysis revealed that total bacterial increment (dcfu) had a highly significant negative direct effect on ammonia concentration ($P < 0.01$, Table 6), indicating microbial growth and proliferation facilitate ammonia conversion, consistent with previous research. In earlier regression models, dcfu showed no conversion effect [17], possibly due to fewer parameters and confounded effects [25], suggesting multiple regression may be less appropriate for multi-factor analysis while path analysis provides more reasonable effect partitioning. Water C:N ratio substantially affects microbial growth; when C:N exceeds 15:1, heterotrophic bacterial growth and ammonia assimilation are promoted, effectively controlling ammonia concentration [36-41]. In NH_4Cl medium, C:N was 15.3:1 ($26.2 \text{ mg} \cdot \text{L}^{-1}$ nitrogen, $400 \text{ mg} \cdot \text{L}^{-1}$ carbon), enabling NT9 to achieve 89.3% ammonia removal (Fig. 2). In simulated aquaculture, C:N was approximately 6:1 [17] based on feed composition (40% crude protein, 5.0% crude fat, 5.0% crude fiber, 15% crude ash, 12% moisture), resulting in carbon limitation and poor ammonia conversion. Cellulase activity (cel) and growth ability (gro) showed negative direct effects (non-significant, $P > 0.05$, Table 6) that nonetheless facilitated ammonia removal by promoting microbial adaptation and growth, while cellulase could enhance cellulose utilization as a prebiotic to promote microbial

growth [42] and reduce ammonia [43]. Amylase activity (amy) also inhibited ammonia accumulation through large indirect effects via cfu (Table 6), related to their highly significant negative correlation ($P < 0.01$, Table 5), though the mechanism requires further investigation. Total bacterial count (cfu) and increment (dcfu) also indirectly promoted ammonia conversion through time (Table 6), while their direct effects were negative, indicating complex nitrogen transformation processes under aquaculture conditions.

In conclusion, *Bacillus* strains NT9 and YB3 possess strong organic nitrogen degradation and ammonia conversion capabilities, but in aquaculture water they primarily promote organic nitrogen degradation with limited ammonia conversion efficiency. Path analysis demonstrated that microbial quantity (cfu) facilitates organic nitrogen degradation (path coefficient 0.550, $P < 0.001$), while microbial proliferation (dcfu) promotes ammonia conversion (path coefficient -0.112, $P < 0.001$). Future research should focus on promoting *Bacillus* growth to achieve synchronous ammonia removal.

References

- [1] Avnimelech Y, Kochba M. Evaluation of nitrogen uptake and excretion by tilapia in bio floc tanks, using ^{15}N tracing[J]. *Aquaculture*, 2009, 287(1): 163-168.
- [2] Piedrahita R H. Reducing the potential environmental impact of tank aquaculture effluents through intensification and recirculation[J]. *Aquaculture*, 2003, 226(1): 35-44.
- [3] Fungesmith S J, Mrp B. Nutrient budgets in intensive shrimp ponds: implications for sustainability[J]. *Aquaculture*, 1998, 164(1-4): 117-133.
- [4] Qi Z X, Li D S, Zhang M P, et al. Experimental studies on nitrogen and phosphorus budgets of shrimp culture pond[J]. *Journal of Fisheries of China*, 1998, 22(2): 124-128 (in Chinese).
- [5] Burford M A, Williams K C. The fate of nitrogenous waste from shrimp feeding[J]. *Aquaculture*, 2001, 198(1): 79-93.
- [6] Lin Y, Chen J. Acute toxicity of ammonia on *Litopenaeus vannamei* Boone juveniles at different salinity levels[J]. *Journal of Experimental Marine Biology and Ecology*, 2001, 259(1): 109-119.
- [7] Ferreira M G P, Melo F P, Lima J P V, et al. Bioremediation and biocontrol of commercial probiotic in marine shrimp culture with biofloc[J]. *Latin American Journal of Aquatic Research*, 2017, 45(1): 167-176.
- [8] Luo G, Wang J, Ma N, et al. Effects of inoculated *Bacillus subtilis* on geosmin and 2-methylisoborneol removal in suspended growth reactors using aquacultural waste for biofloc production[J]. *Journal of Microbiology And Biotechnology*, 2016, 26(8): 1420-1427.
- [9] Elsabagh M, Mohamed R, Moustafa E M, et al. Assessing the impact of

Bacillus strains mixture probiotic on water quality, growth performance, blood profile and intestinal morphology of Nile tilapia, *Oreochromis niloticus*[J]. *Aquaculture Nutrition*, 2018, 24(6): 1613-1622.

[10] Rafael Martinez-Cordova L, Martinez-Porchas M, Coelho Emerenciano M G, et al. From microbes to fish the next revolution in food production[J]. *Critical Reviews in Biotechnology*, 2017, 37(3): 287-295.

[11] Gao J, Gao D, Liu H, et al. Biopotentiality of high efficient aerobic denitrifier *Bacillus megaterium* S379 for intensive aquaculture water quality management[J]. *Journal of Environmental Management*, 2018, 222(9): 1-9.

[12] Xie F X, Zhang F F, Zhou, K, et al. Identification of a *Bacillus amyloliquefaciens* strain and its potential application in water purification[J]. *Acta Scientiae Circumstantiae*, 2012, 32(11): 2781-2788 (in Chinese).

[13] Yang X P, Wang S M, Zhang D W, et al. Isolation and nitrogen removal characteristics of an aerobic heterotrophic nitrifying-denitrifying bacterium, *Bacillus subtilis* A1[J]. *Bioresour Technol*, 2011, 102(2): 854-862.

[14] Yu C H, Wang Y, Guo T, et al. Isolation and identification of ammonia nitrogen degradation strains from industrial wastewater[J]. *Engineering*, 2012, 4: 790-793.

[15] Zhou G J, Zhang X B, Zhu B T, et al. Effects of nitrogen removal in polluted mariculture water containing sediments by *Marichromatium gracile* YL28[J]. *Biotic Resources*, 2017(6): 441-447 (in Chinese).

[16] Zou W S, Liu L G, Zhang J L, et al. Analysis About Effect of Algae-bacteria Immobilized Treat Nitrogen and Phosphorus of Pearl Mussel Aquaculture Wastewater[J]. *Journal of Agro-Environment Science*, 2011, 30(4): 720-725 (in Chinese).

[17] Huang H H, Cheng Q, Lei Y J, et al. Organic nitrogen removal of *Bacillus* strains in water body under low nutrition level[J]. *Acta Scientiae Circumstantiae*, 2018, DOI:10.13671/j.hjkxxb.2018.0322 (in Chinese).

[18] Kora H, Tsuchimoto M, Miyata K, et al. Estimation of body fat content from standard body length and body weight on cultured red sea bream[J]. *Fisheries Science*, 2000, 66(2): 365-371.

[19] Xue B B, Li H, Niu D H, et al. Correlation and path analysis of quantitative traits of new variety of *Sinonovacula constricta* at different months of age[J]. *Journal of Fisheries of China*, 2018, 42(6): 941-949 (in Chinese).

[20] Huang J S, Chen G, Zhang J D, et al. Principal component and path analysis of morphological traits of *Epinephelus fuscoguttatus* at different month ages[J]. *Journal of Fisheries of China*, 2017, 41(7): 1105-1115 (in Chinese).

[21] Huang H H, He L, Lei Y J, et al. Characterization of growth and ammonia removal of a *Bacillus* strain under low nitrogen source condition[J]. *Acta Scientiae Circumstantiae*, 2018, 38(1): 183-192 (in Chinese).

- [22] Li M M. Screening of organic pollutant-degrading bacillus and its clean characterization[D]. Xiamen: Huaqiao University, 2016 (in Chinese).
- [23] Chinese NEPA, 2012. Water and wastewater monitoring methods[M]. Beijing, Chinese Environmental Science Publishing House, 2002: 258-285 (in Chinese).
- [24] Du J J and Chen Z W. Path analysis using linear regression in SPSS[J]. Bulletin of Biology, 2010, 45(2): 4-6 (in Chinese).
- [25] Wan C H and Luo J H. Advanced medical statistics[M]. Beijing: China Science Publishing, 2014: 45-47 (in Chinese).
- [26] Kang P L, Zhang H H, Huang T L, et al. Denitrification characteristics and community structure of aerobic denitrifiers from lake and reservoir sediments[J]. Environmental Science, 2018, 9(5): 1-10 (in Chinese).
- [27] Anand P S S, Kohli M P S, Kumar S, et al. Effect of dietary supplementation of biofloc on growth performance and digestive enzyme activities in *Penaeus monodon*[J]. Aquaculture, 2014, 418(1): 108-115.
- [28] Crab R, Chielens B, Wille M, et al. The effect of different carbon sources on the nutritional value of bioflocs, a feed for *Macrobrachium rosenbergii* post-larvae[J]. Aquaculture Research, 2010, 41(4): 559-567.
- [29] Ebeling J M, Timmons M B, Bisogni J J. Engineering analysis of the stoichiometry of photoautotrophic, autotrophic, and heterotrophic removal of ammonia-nitrogen in aquaculture systems[J]. Aquaculture, 2006, 257(1-4): 346-358.
- [30] Van Wyk P, Davishodgkins M, Laramore R, et al. Farming marine shrimp in recirculating freshwater systems[M]. Tallahassee: Florida department of agriculture and consumer services, 1999: 1-220.
- [31] Crab R, Avnimelech Y, Defoirdt T, et al. Nitrogen removal techniques in aquaculture for a sustainable production[J]. Aquaculture, 2007, 270(1): 1-14.
- [32] Yan F J, Tian X L, Dong S L, et al. Isolation and selection of normal and low temperature bacillus degrading organic pollutants in sea cucumber culturing ponds[J]. Periodical of Ocean University of China, 2013, 43(6): 17-24 (in Chinese).
- [33] Zhang Q H, Feng Y H, Wang J, et al. Study on the characteristics of the ammonia-nitrogen and residual feeds degradation in aquatic water by *Bacillus licheniformis*[J]. Acta Hydrobiologica Sinica, 2011, 35(3): 498-503 (in Chinese).
- [34] Cardona E, Gueguen Y, Magre K, et al. Bacterial community characterization of water and intestine of the shrimp *Litopenaeus stylirostris* in a biofloc system[J]. BMC Microbiology, 2016, 16: DOI 10.1186/s12866-016-0770-z.
- [35] Yu Y X, Jiang Y, Zhang Z, et al. The microflora changes of rotifer and artemia with probiotics addition in *Scophthalmus maximus* larva breeding[J].

Journal of Fisheries of China, 2017, 41(4): 602-612 (in Chinese).

[36] Avnimelech Y. Carbon/nitrogen ratio as a control element in aquaculture systems[J]. Aquaculture, 1999, 176(3): 227-235.

[37] Lu B G, Wang H Y, Xie J, et al. Effect of C/N ratio on bioflocs formation and water quality in zero-water exchange grass carp tanks[J]. Journal of Fisheries of China, 2013(8): 1220-1228 (in Chinese).

[38] Zheng X, Zhang D, Qin J, et al. The effect of C/N ratio on bacterial community and water quality in a mussel-fish integrated system[J]. Aquaculture Research, 2018, 49(4): 1699-1708.

[39] Wang G, Yu E, Xie J, et al. Effect of C/N ratio on water quality in zero-water exchange tanks and the biofloc supplementation in feed on the growth performance of crucian carp, *Carassius auratus*[J]. Aquaculture, 2015, 443(6): 98-104.

[40] Xu W J, Morris T C, Samocha T M. Effects of two commercial feeds for semi-intensive and hyper-intensive culture and four C/N ratios on water quality and performance of *Litopenaeus vannamei* juveniles at high density in biofloc-based, zero-exchange outdoor tanks[J]. Aquaculture, 2018(3), 490: 194-202.

[41] Xu W J, Morris T C, Samocha T M. Effects of C/N ratio on biofloc development, water quality, and performance of *Litopenaeus vannamei* juveniles in a biofloc-based, high-density, zero-exchange, outdoor tank system[J]. Aquaculture, 2016, 453(2): 169-175.

[42] Qin Y, Hou J, Deng M, et al. Bacterial abundance and diversity in pond water supplied with different feeds[J]. Scientific Reports, 2016, 6: 35232.

[43] Deng M, Chen J, Hou J, et al. The effect of different carbon sources on water quality, microbial community and structure of biofloc systems[J]. Aquaculture, 2018, 482(1): 103-110.

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Note: Figure translations are in progress. See original paper for figures.

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