ISOLATION AND FUNCTIONAL EVALUATION OF *Dirkmeia churashimaensis* FROM AQUACULTURE PONDS FOR EFFICIENT NITRITE AND AMMONIA REMOVAL AND PROBIOTIC APPLICATION

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Abstract

This study focused on the isolation and characterization of a novel microorganism with the capacity to remove nitrite and ammonia from aquaculture environments, aiming to improve water quality. Among several microbial consortia screened, the FW4' group exhibited superior nitrite removal efficiency, from which 20 bacterial strains were successfully isolated. The most effective isolate, FW4'-T3, completely eliminated nitrite-N within 72 h (Fig. 2).

Morphological and molecular analyses confirmed that FW4'-T3 was identified as D. churashimaensis, showing 99.86% sequence similarity with reference strains (Fig. 4). Safety assessments in multiple aquaculture species demonstrated its non-pathogenic nature, supporting its potential application in aquaculture systems.

Nitrogen removal efficiency was significantly influenced by carbon source and carbon-to-nitrogen (C/N) ratio. Sucrose outperformed glucose, achieving complete nitrogen removal within 24 h at C/N ratios of 15 and 20. Optimal performance was observed at 30˚C in NaCl-free conditions. Statistical analysis further confirmed that salinity, temperature, and incubation time significantly affected nitrogen removal, with synergistic interactions between salinity and temperature.

Results

Table 1. Assessment of safety and environmental conditions of aquatic animals after a 7-day immersion challenge with *Dirkmeia churashimaensis* 768 FW4'-T3. F: Freshwater; S: Saltwater (35 psu).

Categories	Common name	Organism	Body weight (g)	Environment	Mortality (%)
Fish	Malawi hawk cichlid	*Aristochromis christyi*	0.09 ± 0.001	F	0
	Common carp	*Cyprinus carpio*	1.44 ± 0.05	F	0
	Hump-head	*Cyrtocara moorii*	0.13 ± 0.01	F	0
	Milk fish	*Chanos chanos*	1.49 ± 0.05	S	0
	Speckled blue grouper	*Epinephelus cyanopodus*	97.00 ± 19.11	S	0
	Blue-and-yellow grouper	*E. flavocaeruleus*	115.01 ± 13.66	S	0
	Suckermouth catfish	*Hypostomus plecostomus*	0.55 ± 0.03	F	0
	Tilapia	*Oreochromis* sp.,	2.41 ± 0.08	F	0
	Guppy	*Poecilia reticulata*	0.21 ± 0.01	F	0
	Blunthead cichlid	*Tropheus moorii*	2.22 ± 0.11	F	0
Crustaceans	Red claw crayfish	*Cherax quadricarinatus*	0.49 ± 0.02	F	0
	White shrimp	*Penaeus vannamei*	5.33 ± 0.16	S	0
	Tiger shrimp	*P. monodon*	0.03 ± 0.001	S	0
	Spiny lobster	*Panulirus homarus*	27.33 ± 1.97	S	0
Bivalves	Iron clam	*Cyclina sinensis*	32.56 ± 1.17	S	0
	Portuguese oyster	*Crassostrea angulata*	129.66 ± 8.65	S	0

Table 2. Components in N media with varying C/N ratios for the evaluation of nitrite N and ammonia-N reduction in water.

Components	C/N ratio		
	10	15	20
Glucose / Sucrose (g/L)	0.1250 / 0.1191	0.1875 / 0.1786	0.2500 / 0.2381
KH_2PO_4 (g/L)	2	2	2
$MgSO_4 \cdot 7H_2O$ (g/L)	0.05	0.05	0.05
$FeSO_4 \cdot 7H_2O$ (g/L)	0.02	0.02	0.02

Table 3. Tolerance of *Dirkmeia churashimaensis* FW4'-T3 to varying temperature and NaCl concentrations. -: no observable growth; +: colony diameter 1 mm.

Temperature (°C)							
10	20	25	28	30	35	40	50
-	+	++	++	+++	++	-	-
NaCl (%)							
0.5	10	15	20	30	40	50	60
++	++	++	++	++	++	++	++

Table 4. Effect of carbon sources and C/N ratios on nitrite-N reduction of *Dirkmeia churashimaensis* FW4'-T3. The data are presented as mean ± standard error (S.E.). Within each carbon source, different superscript letters (a, b, c) denote statistically significant differences among C/N ratios, whereas within each C/N ratio, different superscript letters (x, y) indicate significant differences between carbon sources. Statistical significance was determined at $p < 0.05$.

Carbon source	C/N ratio	Nitrite-N concentration (mg/L) after inoculation (h)					
		0	6	12	24	48	72
Glucose	10	5.07 ± 0.02 ^{a,x}	4.31 ± 0.11 ^{a,x}	2.71 ± 0.08 ^{a,x}	2.01 ± 0.03 ^{a,x}	1.82 ± 0.02 ^{a,x}	1.21 ± 0.61 ^{a,x}
	15	5.14 ± 0.06 ^{a,x}	4.15 ± 0.12 ^{a,x}	2.15 ± 0.05 ^{b,x}	0.92 ± 0.13 ^{b,x}	0.41 ± 0.26 ^{b,x}	0.03 ± 0.03 ^{b,x}
	20	5.15 ± 0.12 ^{a,x}	4.13 ± 0.08 ^{a,x}	2.02 ± 0.09 ^{b,x}	0.13 ± 0.08 ^{c,x}	0.00 ± 0.00 ^{c,x}	0.00 ± 0.00 ^{b,x}
Sucrose	10	5.03 ± 0.01 ^{a,x}	4.14 ± 0.13 ^{a,x}	2.40 ± 0.16 ^{a,y}	1.05 ± 0.17 ^{a,y}	0.74 ± 0.25 ^{a,y}	0.51 ± 0.36 ^{a,x}
	15	5.09 ± 0.06 ^{a,x}	4.13 ± 0.01 ^{a,x}	2.33 ± 0.15 ^{a,x}	0.00 ± 0.00 ^{b,y}	0.00 ± 0.00 ^{b,x}	0.00 ± 0.00 ^{b,x}
	20	5.01 ± 0.10 ^{a,x}	4.23 ± 0.09 ^{a,x}	2.52 ± 0.21 ^{a,y}	0.00 ± 0.00 ^{b,y}	0.00 ± 0.00 ^{b,x}	0.00 ± 0.00 ^{b,x}

Table 5. Effect of carbon source and C/N ratio on ammonia-N reduction of *Dirkmeia churashimaensis* FW4'-T3. The data are presented as mean ± standard error (S.E.). Within each carbon source, different superscript letters (a, b, c) denote statistically significant differences among C/N ratios, whereas within each C/N ratio, different superscript letters (x, y) indicate significant differences between carbon sources. Statistical significance was determined at $p < 0.05$.

Carbon sources	C/N ratios	Ammonia-N concentration (mg/L) after inoculation (h)					
		0	6	12	24	48	72
Glucose	10	10	4.96 ± 0.14 ^{a,x}	3.96 ± 0.02 ^{a,x}	0.73 ± 0.14 ^{a,x}	0.09 ± 0.08 ^{a,x}	0.00 ± 0.00 ^{a,x}
	15	15	4.99 ± 0.05 ^{a,x}	4.02 ± 0.05 ^{a,x}	0.00 ± 0.00 ^{b,x}	0.00 ± 0.00 ^{b,x}	0.00 ± 0.00 ^{a,x}
	20	20	4.92 ± 0.03 ^{a,x}	4.06 ± 0.16 ^{a,x}	0.00 ± 0.00 ^{b,x}	0.00 ± 0.00 ^{b,x}	0.00 ± 0.00 ^{a,x}
Sucrose	10	10	4.96 ± 0.12 ^{a,x}	4.19 ± 0.07 ^{a,x}	0.17 ± 0.04 ^{a,y}	0.00 ± 0.00 ^{a,y}	0.00 ± 0.00 ^{a,x}
	15	15	5.01 ± 0.02 ^{a,x}	3.97 ± 0.03 ^{b,x}	0.00 ± 0.00 ^{b,x}	0.00 ± 0.00 ^{a,x}	0.00 ± 0.00 ^{a,x}
	20	20	4.99 ± 0.08 ^{a,x}	3.89 ± 0.12 ^{b,x}	0.00 ± 0.00 ^{b,x}	0.00 ± 0.00 ^{a,x}	0.00 ± 0.00 ^{a,x}

Table 6. Under a C/N ratio of 15, with sucrose as carbon sources, *Dirkmeia churashimaensis* FW4'-T3 effectively reduces the nitrite-N concentration in the medium following inoculation. Different letters within each NaCl level indicate significant differences among different temperature (a, b, c), while within each temperature, different letters indicate significant differences between NaCl levels (x, y). The significance level was determined at $p<0.05$.

NaCl (%)	Temperature (°C)	Nitrite-N concentration (mg/L) after inoculation (h)					
		0	6	12	24	48	72
0	20	5.06 ± 0.02 ^{a,x}	5.03 ± 0.04 ^{a,x}	4.55 ± 0.06 ^{a,y}	2.94 ± 0.09 ^{a,y}	0.00 ± 0.00 ^{a,x}	0.00 ± 0.00 ^{a,x}
	25	5.04 ± 0.14 ^{a,x}	4.95 ± 0.07 ^{ab,x}	4.08 ± 0.06 ^{b,y}	0.27 ± 0.10 ^{b,y}	0.00 ± 0.00 ^{a,x}	0.00 ± 0.00 ^{a,x}
	30	5.06 ± 0.08 ^{a,x}	4.88 ± 0.03 ^{b,x}	2.99 ± 0.08 ^{c,y}	0.00 ± 0.00 ^{c,y}	0.00 ± 0.00 ^{a,x}	0.00 ± 0.00 ^{a,x}
3.5	20	5.07 ± 0.07 ^{a,x}	5.01 ± 0.07 ^{a,x}	4.91 ± 0.03 ^{a,x}	3.93 ± 0.03 ^{a,x}	0.00 ± 0.00 ^{a,x}	0.00 ± 0.00 ^{a,x}
	25	5.02 ± 0.09 ^{a,x}	5.05 ± 0.07 ^{a,x}	4.69 ± 0.05 ^{b,x}	2.18 ± 0.11 ^{b,x}	0.00 ± 0.00 ^{a,x}	0.00 ± 0.00 ^{a,x}
	30	5.09 ± 0.06 ^{a,x}	5.02 ± 0.12 ^{a,x}	4.18 ± 0.07 ^{c,x}	0.00 ± 0.00 ^{c,x}	0.00 ± 0.00 ^{a,x}	0.00 ± 0.00 ^{a,x}

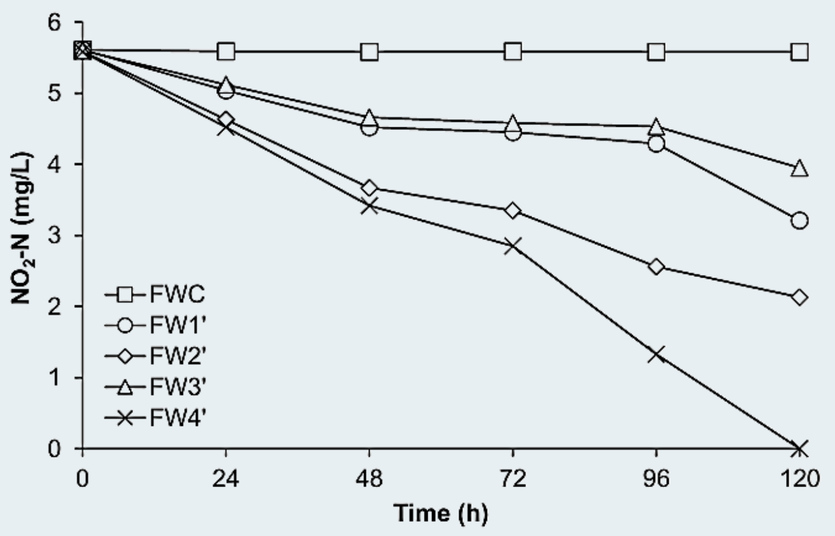


Figure 1. Reduction of nitrite-N in media inoculated with water samples from four prawn ponds. FWC: control medium without inoculation; FW1'-FW4': media inoculated with water samples from different prawn ponds.

Table 7. Variations in the relative gene expression of nitrite reductase in *Dirkmeia churashimaensis* FW4'-T3 across different carbon sources and C/N ratios. Different letters within each carbon source indicate significant differences among various C/N ratios (a, b, c), while within each C/N ratio, different letters indicate significant differences between carbon sources (x, y). The significance level was determined at $p<0.05$.

Carbon source	C/N ratio	Relative gene expression of nitrite reductase after inoculation (h)					
		0	6	12	24	48	72
Glucose	10	1.0 ± 0.4 ^{a,x}	165.0 ± 4.6 ^{c,x}	507.0 ± 29.4 ^{c,x}	357.1 ± 41.1 ^{b,y}	733.7 ± 141.2 ^{a,x}	1932.2 ± 331.1 ^{a,x}
	15	1.0 ± 0.4 ^{a,x}	738.2 ± 93.1 ^{a,x}	850.4 ± 34.2 ^{b,x}	1705.5 ± 122.0 ^{a,x}	12.2 ± 1.0 ^{b,y}	404.2 ± 27.9 ^{b,x}
	20	1.0 ± 0.4 ^{a,x}	491.3 ± 77.9 ^{b,x}	1206.2 ± 117.6 ^{a,y}	1878.3 ± 137.8 ^{a,x}	19.9 ± 4.3 ^{b,y}	35.9 ± 5.0 ^{c,x}
Sucrose	10	1.0 ± 0.4 ^{a,x}	240.6 ± 37.7 ^{b,x}	214.9 ± 27.8 ^{b,y}	1178.5 ± 92.2 ^{b,x}	928.2 ± 117.7 ^{a,x}	92.4 ± 5.3 ^{a,y}
	15	1.0 ± 0.4 ^{a,x}	730.9 ± 149.3 ^{a,x}	782.7 ± 7.2 ^{b,x}	1772.2 ± 150.5 ^{a,x}	124.5 ± 9.9 ^{b,x}	39.5 ± 2.5 ^{b,y}
	20	1.0 ± 0.4 ^{a,x}	729.4 ± 164.8 ^{a,x}	2078.4 ± 398.9 ^{a,x}	1704.6 ± 363.2 ^{a,x}	32.8 ± 2.8 ^{b,x}	14.3 ± 1.4 ^{c,y}

Table 8. Under a C/N ratio of 15, with sucrose as carbon sources, *Dirkmeia churashimaensis* FW4'-T3 reduces the ammonia-N concentration in the medium following inoculation. Different letters within each NaCl level indicate significant differences among different temperature (a, b, c), while within each temperature, different letters indicate significant differences between NaCl levels (x, y, z). The significance level was determined at $p < 0.05$

NaCl (%)	Temperature (°C)	Ammonia-N concentration (mg/L) after inoculation (h)					
		0	6	12	24	48	72
0	20	5.05 ± 0.16 ^{a,x}	4.22 ± 0.15 ^{a,y}	1.94 ± 0.29 ^{a,y}	0.00 ± 0.00 ^{a,x}	0.00 ± 0.00 ^{a,x}	0.00 ± 0.00 ^{a,x}
	25	4.98 ± 0.09 ^{a,x}	3.98 ± 0.06 ^{a,y}	0.30 ± 0.14 ^{b,y}	0.00 ± 0.00 ^{a,x}	0.00 ± 0.00 ^{a,x}	0.00 ± 0.00 ^{a,x}
	30	4.88 ± 0.12 ^{a,x}	3.16 ± 0.14 ^{b,y}	0.03 ± 0.01 ^{c,y}	0.00 ± 0.00 ^{a,x}	0.00 ± 0.00 ^{a,x}	0.00 ± 0.00 ^{a,x}
35	20	5.04 ± 0.17 ^{a,x}	4.65 ± 0.12 ^{a,x}	3.79 ± 0.10 ^{a,x}	0.00 ± 0.00 ^{a,x}	0.00 ± 0.00 ^{a,x}	0.00 ± 0.00 ^{a,x}
	25	4.94 ± 0.17 ^{a,x}	4.34 ± 0.15 ^{b,x}	2.91 ± 0.20 ^{b,x}	0.00 ± 0.00 ^{a,x}	0.00 ± 0.00 ^{a,x}	0.00 ± 0.00 ^{a,x}
	30	4.98 ± 0.09 ^{a,x}	4.11 ± 0.01 ^{b,x}	0.53 ± 0.30 ^{c,x}	0.00 ± 0.00 ^{a,x}	0.00 ± 0.00 ^{a,x}	0.00 ± 0.00 ^{a,x}

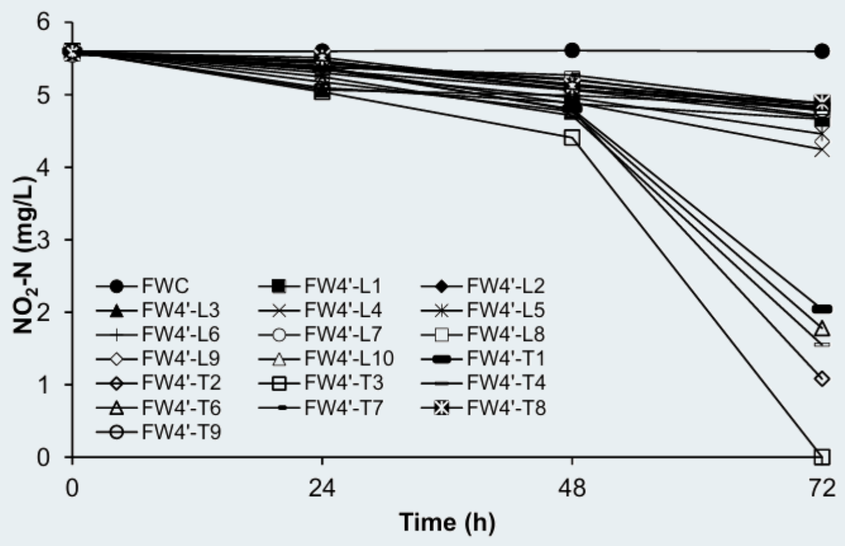


Figure 2. A comparative analysis of nitrite-N reduction by isolates derived from FW4' samples. FWC: control medium without microorganism inoculation.

Conclusion

Based on the results of this study, *D. churashimaensis* FW4'-T3 was identified as a highly effective nitrite- and ammonia-N-reducing yeast strain with strong potential for application in aquaculture water quality management. The strain demonstrated complete nitrite-N removal under optimal C/N ratios (≥15), particularly when sucrose was used as the carbon source. Its efficacy was closely linked to the upregulation of nitrite reductase gene expression, which was enhanced under higher C/N conditions. Furthermore, FW4'-T3 exhibited robust nitrogen-removal capacity across a broad salinity (0–3.5%) and temperature (20–30 ˚C) range, aligning with its optimal growth conditions. The strain was also shown to be non-pathogenic to a range of aquatic species, underscoring its safety for practical use. Collectively, these findings highlight *D. churashimaensis* FW4'-T3 as a promising probiotic candidate for mitigating nitrogenous waste in intensive aquaculture systems.

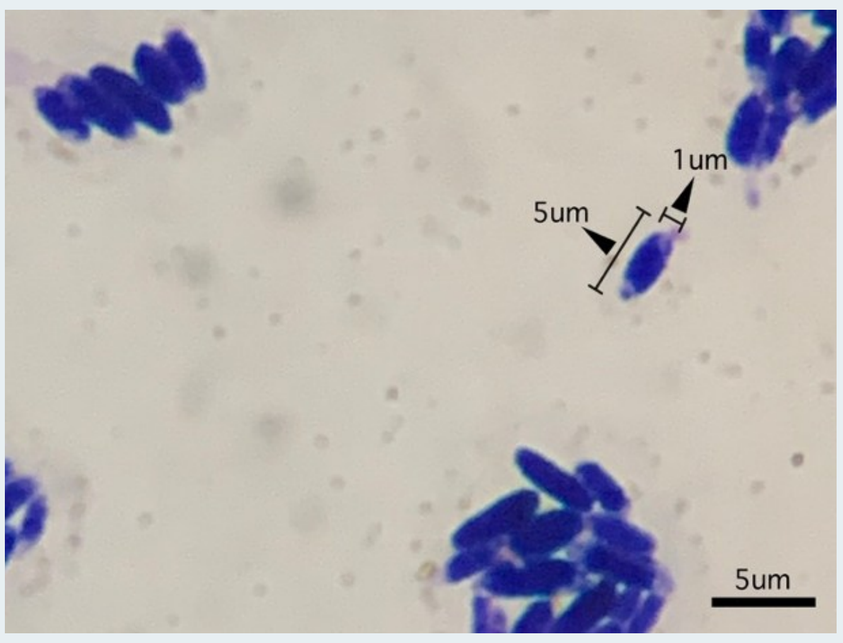


Figure 3. Microscopic image of *Dirkmeia churashimaensis* FW4'-T3 isolated cells displayed for observation.

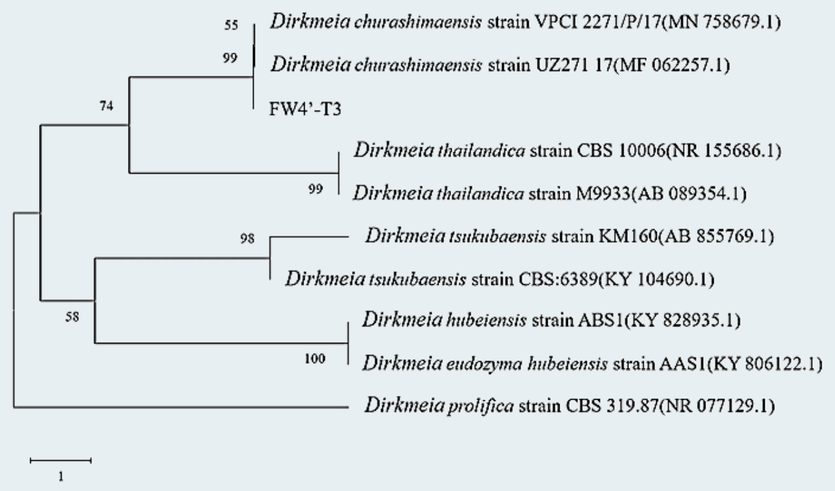


Figure 4. Phylogenetic tree constructed based on ITS sequences from various *Dirkmeia* species. The tree was generated using the neighbor-joining method, with bootstrap values derived from 1000 replicates provided for each internal node. The tree is scaled with uniform branch lengths, and evolutionary distances are represented accordingly.