Optimal nitrogen source for the recovery of phycoerythrin in discolored Rhodomonas sp.

Optimal nitrogen source for the recovery of phycoerythrin in discolored *Rhodomonas* sp.

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Abstract

Rhodomonas species are of interest as excellent aquaculture feed because of their ideal biochemical composition, especially their high protein content, resulting from the presence of intracellular phycoerythrin (PE). One of the most crucial issue with this species as a feed source is PE degradation under ambient nitrogen starvation, which leads to unpredictable growth defects and reduced nutritional value associated with cell

discoloration. The aim of this study was to propose an optimal nitrogen source for the recovery of intracellular PE in discolored *Rhodomonas* sp. We supplied three different nitrogen sources, i.e., nitrate, ammonium, and urea, to PE-degraded cells and monitored changes in PE content and the expression of related genes. Supplementation with each nitrogen source increased cell density, with nitrate exhibiting the highest specific growth rate of 0.30 ± 0.02 day^{-1}. The maximum PE content in each nitrogen group at 48 h after N supplementation was about 3.8 pg $cell^{-1}$ with no major difference between groups. In contrast, the highest relative PE recovery rate of 5.73 ± 0.18-fold and the highest PE productivities of 0.32 ± 0.01 μg mL^{-1} h^{-1} were obtained in the nitrate group. Nitrogen transporter genes, including *RhNrt2*, *RhAmt1*, and *RhDur3*, were upregulated under nitrogen-starvation conditions, demonstrating the ability of this species to utilize each of the tested nitrogen sources. Furthermore, *RhCpeb,* which serves as a molecular indicator of PE biosynthesis, was significantly upregulated in all nitrogen-supplemented groups, with the strongest and fastest increase in expression in the nitrate group, emphasizing the potential of nitrate as the most favorable nitrogen source for PE recovery in this species.

Our findings pave the way for informed strategies for the stable management and production of *Rhodomonas* species as high-protein feed in aquaculture.

Keywords

Rhodomonas, nitrogen source, nitrogen starvation, nitrogen transporter, phycoerythrin, gene expression

1. Introduction

Species of the cryptophyte genus *Rhodomonas*, such as *R. salina*, *R. baltica, R. reticulata*, and *R. lens*, are excellent aquaculture feeds because of their ideal biochemical compositions [1–3]. Many studies have been undertaken to optimize culture conditions and enhance nutritional value [4–8]. Consequently, *Rhodomonas* species are now used as feed for diverse marine animals, such as filter-feeder mollusks, crustaceans, and zooplankton, as live prey [9–11].

A notable aspect of the nutritional value of *Rhodomonas* species compared to other microalgae is their exceptionally high protein content. Protein is an essential dietary component, and *Rhodomonas* species are therefore commonly recognized as excellent high-protein feeds for marine animals. For example, in a recent study by Coutinho et al. [18], *R. lens* was described as a "Premium" microalgae for enriching rotifers due to its high protein content. The high protein content in *Rhodomonas* species is mainly due to the presence of phycoerythrin (PE), which has a bright red color and is the major phycobiliprotein in this species, in the cells [12,13]. This phycobiliprotein acts as a photosynthetic light-harvesting pigment that provides energy to photosystem II [14–16].

Consisting of an α1α2ββ heterodimeric protein-pigment complex with a molecular weight of 24 kDa, PE represents over 20% of the intracellular protein content of cryptophytes [14,17]. Therefore, increasing the intracellular PE content enhances the nutritional benefits of *Rhodomonas* species as a feed, i.e. the protein content, and this characteristic justifies research into improving the culture protocol for PE production in this species. Our previous study found a correlation between protein and PE content in *Rhodomonas* sp. (Hf-1 strain) and achieved an increase in protein content to more than 60% of the dry weight through enhanced PE biosynthesis [19].

Despite the noted advantages of *Rhodomonas* species as feed, unresolved issues must be addressed for its effective use. The most crucial of these are the unpredictable growth defects and reduced nutritional value associated with cell discoloration. This phenomenon in cryptophytes has been documented by several researchers since the 1970s and is known to be due to intracellular PE degradation caused by ambient nitrogen (N) starvation [17,20,21]. Da Silva et al. [17] reported that the PE content in *Rhodomonas* sp. reduced by up to 90% under N starvation, inducing a rapid change in culture color from bright red to yellow. Our previous study also showed that N starvation leads to a 75% reduction in

PE content in *Rhodomonas* sp., resulting in a reduced growth rate and nutritional value, with a drastic change in cell color [19]. Lichtlé [20] speculated that phycobiliproteins are biosynthesized as an internal N reserve under N-sufficient conditions, whereas under N starvation, they are autolyzed and used as protein substrates to synthesize other proteins essential for survival. Several studies support this hypothesis [17,21–23].

Some studies have focused on enhancing PE biosynthesis in *Rhodomonas* species by applying environmental stimuli. For instance, Latsos et al. [24,25] reported the effects of optimal light conditions, salinity, and pH on increasing the PE content in *Rhodomonas* sp. Chaloub et al. [21] investigated the combined effect of irradiance, temperature, and nitrate concentration on PE content in *Rhodomonas* sp. and proposed that PE biosynthesis was enhanced under low light intensity at 26°C. However, considering the practical operation of this species as a feed in aquaculture, more crucial information should be provided on methodologies to recover discolored cells to a sound state rather than techniques to produce excessive PE. This is because PE degradation occurs in many aquaculture situations, including preservation, transport, and production, and its prediction remains

84 difficult. Lichtlé [20] reported that PE reappears when N deficiency is stopped; however,
85 further updates in this respect are limited in the literature.
86 The aim of this study was to propose an optimal N source for the recovery of PE from
87 discolored *Rhodomonas* sp. Although *Rhodomonas* species can utilize different forms of
88 N, including inorganic and organic N, for growth [26–28], few studies have investigated
89 their effects on PE biosynthesis. In this study, we supplied three different N sources, i.e.,
90 nitrate, ammonium, and urea, to PE-degraded cells and monitored the changes in
91 intracellular PE content to assess the effect of their supplementation on PE recovery. The
92 uptake of each N source by *Rhodomonas* sp. was verified by expression analysis of N
93 transporters, including the nitrate transporter gene (*RhNrt2*), ammonium transporter gene
94 (*RhAmt1*) and urea transporter gene (*RhDur3*). In addition, the expression of the *RhCpeb*,
95 encoding the β-subunit of PE and identified in our previous report as a useful molecular
96 indicator of PE biosynthesis [29], was monitored to assess the PE biosynthetic activity
97 under different N sources. Our findings contribute to the stable management and
98 production of *Rhodomonas* species as high-protein feeds in aquaculture.

99 **2. Materials and methods**

100 *2.1 Strain and experimental set-up*

101 The *Rhodomonas* sp. used in this study has been described previously [19]. *Rhodomonas*
102 sp. was subcultured in Guillard 2F medium [30] more than three times prior to the
103 experiment. Cultures were maintained at 20°C under continuous (24L:0D) white light
104 emitting diodes at 80 μmol m^{-2} s^{-1} with a sufficient filtered airflow (0.2 μm pore size) to
105 agitate the culture medium.

106 Batch culture experiments were conducted using three N sources, i.e., nitrate, ammonium,
107 and urea, to investigate the effects of different N sources on PE recovery. Starter cultures
108 were grown in 1,000 mL Erlenmeyer flasks containing Guillard 2F medium. After 3 days
109 of culture, the medium was replaced with N-free medium according to the following
110 procedure. Algal cells were collected by centrifugation at 1,000 × *g* for 10 min, washed
111 with sterilized N-free seawater to remove residual N, and resuspended in N-free Guillard
112 2F medium (the absence of N was confirmed by the colorimetric method). After 2 days,
113 the cell suspension became discolored. Subsequently, $NaNO_3$, $(NH_4)_2SO_4$, and CH_4N_2O
114 (> 99%, FUJIFILM Wako chemicals, Osaka, Japan) were separately supplied at a final N
115 concentration of 7.0 mM, mirroring Guillard 4F (nitrate, ammonium, and urea groups).

Medium without added N was used as a control. Cultures were maintained at 20°C under continuous white light emitting diodes at 80 μmol m^{-2} s^{-1} with a sufficient filtered airflow (0.2 μm pore size). Three replicate flasks were prepared for each treatment group. The algal cells were sampled every 12 h after N-source supplementation for cell counting, PE measurements, and RNA extraction.

Another batch culture was prepared with N-sufficient (7 mM of either $NaNO_3$, $(NH_4)_2SO_4$, or CH_4N_2O) and N-starved (0 mM of any N source) conditions to investigate the expression of the N transporter genes *RhNrt2*, *RhAmt1*, and *RhDur3* under different N conditions. The culture conditions were the same as those described above. Algal cells in the N-sufficient group were sampled 12 h after supplementation with each N source, whereas those in the N-starved groups were sampled 72 h after subjecting the cells to N starvation for 72 h to eliminate the effects of intracellular N metabolites. The cells were used for RNA extraction.

2.2 Cell growth and PE measurements

The cells were counted by direct observation under an optical microscope (BX51, Olympus, Tokyo, Japan) using a Neubauer counting chamber (ERMA, Tokyo, Japan).

132 The specific growth rates (μ, day^{-1}) were calculated from cell counts using the following

133 equation:

134 $$\mu = \frac{\ln (N_2/N_1)}{t_2 - t_1}$$

135 where N_1 and N_2 are the cell densities at times t_1 and t_2, respectively, and t_1 and t_2 are the

136 initial and final measurements in the exponential growth phase, respectively.

137 PE extraction was performed by a modified method of our previous study [19].

138 Microalgal pellets were obtained from 20 mL cell suspensions by centrifugation at 2,000

139 × *g* for 10 min to determine the PE content per cell. Pellets immersed in 5 mL of phosphate

140 buffer (0.1 M, pH = 6) (FUJIFILM Wako chemicals) were stored at −35°C for 24 h to

141 disrupt cells, then at 5°C for 24 h to extract PE. The extracts were centrifuged at 12,000

142 × *g* for 5 min to remove cell debris. The absorbance (*A*) of the resulting extracts was

143 measured at 455, 564, 592, and 750 nm using a spectrophotometer (UV-1800, Shimadzu

144 Biotech, Kyoto, Japan), with phosphate buffer as a blank. All absorbance values were

145 scatter-corrected by subtracting the absorbance at 750 nm. PE content (pg $cell^{-1}$) was

146 calculated using the following equation: [31]

147 $$PE\ (pg\ cell^{-1})$$

148 $$= \{[(A_{564} - A_{592}) - (A_{455} - A_{592}) \times 0.2] \times 0.12\} \times V_{buffer}$$

149 $$\times\ cell\ density^{-1} \times 10^{-9}$$

150 where V_{buffer} is the buffer volume and *cell density* is the total number of cells in 20 mL of

151 the harvested cell suspension. The relative PE recovery rate (R_{PE}) was calculated based

152 on the PE contents using the following equation:

153 $$R_{PE} = \frac{PE_t}{PE_i}$$

154 where PE_t is the PE content at time t and PE_i is the initial PE content immediately before

155 each N source is supplemented. Finally, PE productivities ($P_{PE,}$ μg mL^{-1} h^{-1}) were

156 calculated using the following equation:

157 $$P_{PE}(\mu g\ mL^{-1}\ hour^{-1}) = \frac{PE_2 \times N_2 - PE_1 \times N_1}{t_2 - t_1}$$

158 where PE_1 and PE_2 are the PE contents and N_1 and N_2 are the cell densities at times t_1 and

159 $t_{2,}$ respectively.

160 *2.3 RNA extraction and cDNA synthesis*

161 Algal cells were harvested by centrifugation at 12,000 × *g* for 5 min from 2 mL samples,

162 and cell pellets were immediately frozen and stored at −80°C until RNA extraction. Total

163 RNA was extracted using NucleoSpin RNA Plant (Macherey-Nagel, Düren,
164 Germany) following the manufacturer's instructions. The RNA concentration and purity
165 were determined using a Biospec-Nano spectrophotometer (Shimadzu Biotech) at
166 260/280 nm (A_{260}/A_{280}). cDNA synthesis was performed with 0.3 μg of total RNA using
167 a PrimeScript II 1st strand cDNA Synthesis Kit (Takara Bio Inc., Shiga, Japan) with
168 random hexamer primers following the manufacturer's instructions. The synthesized
169 cDNA was used for expression analysis of *RhCpeb* and N transporter genes and of the
170 *RhAmt1* and *RhDur3* genes. The procedures and results for gene identification of *RhAmt1*
171 and *RhDur3*, including cloning and resequencing analyses, are provided in the
172 Supplementary file. The details of *RhCpeb* and *RhNrt2* have been described in our
173 previous study [29].

174 *2.4 RT-qPCR analysis*

175 Expression analyses of *RhCpeb* and the N transporter genes *RhNrt2*, *RhAmt1*, and
176 *RhDur3* were performed. Gene-specific primers used for RT-qPCR are listed in Table 1.
177 RT-qPCR reactions were performed using a Lightcycler 96 (Roche Diagnostics, Ottweiler,
178 Germany) and TB Green Premix Ex Taq II (Takara Bio Inc.) following the manufacturer's

179 instructions. The reaction mix contained 10 µL of TB Green Premix Ex Taq II, 2.0 µL of
180 diluted cDNA template, and 0.8 µL each of forward and reverse primers (final primer
181 concentration was 400 nM) in a final volume of 20 µL. Thermocycler conditions were
182 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 60°C for 30 s, and 60°C for 60 s.
183 All reactions were performed in duplicate. The crossing cycle number (Cp) was
184 automatically determined for each reaction by the Lightcycler 96 application software,
185 with default parameters determined using the second derivative method. Gene expression
186 was analyzed using the Pfaffl method [32], and *ACT* was used as an internal control for
187 normalization [29].

Table 1 Nucleotide sequence of the primers designed for RT-qPCR analysis.

Target gene	Sequence (5′-3 ′)	Product size (bp)	*E* (%)	R^2	Source
RhCpeb	AGCCATACGACGGTTAGTGTAG	185	94.5	1.000	[29]
	TAGGTGGTGCAGATCTACAAGC				
Act	TTCTCCTTGATGTCCCGCAC	122	103.3	0.993	[29]
	ATCCTCCGTCTTGACCTTGC				
RhNrt2	GCTCTTCTTCATCGCAGTGAAC	180	105.2	0.998	[29]
	GTTCGTTTCTTCATCTCGCTCG				
RhAmt1	AGCCGAACCACAGGATGAAC	139	100.8	1.000	This study
	GGGTTCTCGGGTTTGATGGG				
RhDur3	TGACGGGCATGCTTATCCTC	137	96.6	0.997	This study
	GTAGTAGGTGCCCTTCAGGC				

Primer efficiency E (%) = $(10^{-1/\text{slope}} - 1) \times 100$

188

189 *2.5 Statistical analyses*

190 Results were expressed as means of three biological replicates ± SE. Data were analyzed
191 using Tukey's test for specific growth rate and PE productivity and one-way ANOVA,
192 followed by Dunnett's multiple comparison test for relative gene expression. The
193 significance threshold was set at $P < 0.05$. All the statistical analyses were performed
194 using Microsoft Excel.

195 **3. Results**

196 *3.1 Cell growth and PE contents under different N sources*

197 Fig. 1 shows the changes in the cell density of *Rhodomonas* sp. grown using different N
198 sources. The cell density in each group at 0 h, when each N source was supplemented,
199 was $2.7 \pm 0.01 \times 10^6$ cells mL^{-1}. The N-supplemented groups showed an exponential
200 increase in cell density from 12 h of the experiment onward, and the maximum cell
201 density in each group was 4.5×10^6 cells mL^{-1} in the nitrate group, 3.9×10^6 cells mL^{-1}
202 in the ammonium group, and 3.9×10^6 cells mL^{-1} in the urea group. The highest specific
203 growth rate of 0.30 ± 0.02 day^{-1} was obtained in the nitrate group, followed by $0.26 \pm$
204 0.03 day^{-1} in the urea group, and 0.23 ± 0.03 day^{-1} in the ammonium group (Table 2). In

contrast, in the control group without added N sources, cell density barely increased throughout the experiment.

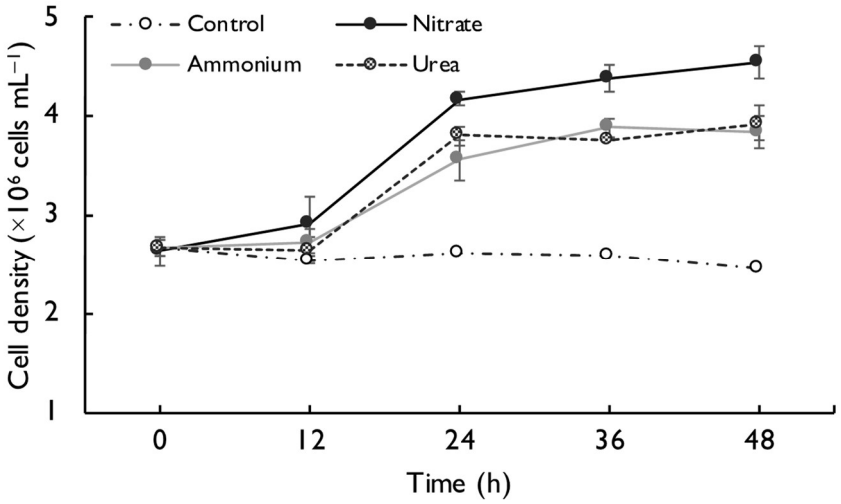


Fig. 1. Changes in cell density of *Rhodomonas* sp. grown using different nitrogen sources.

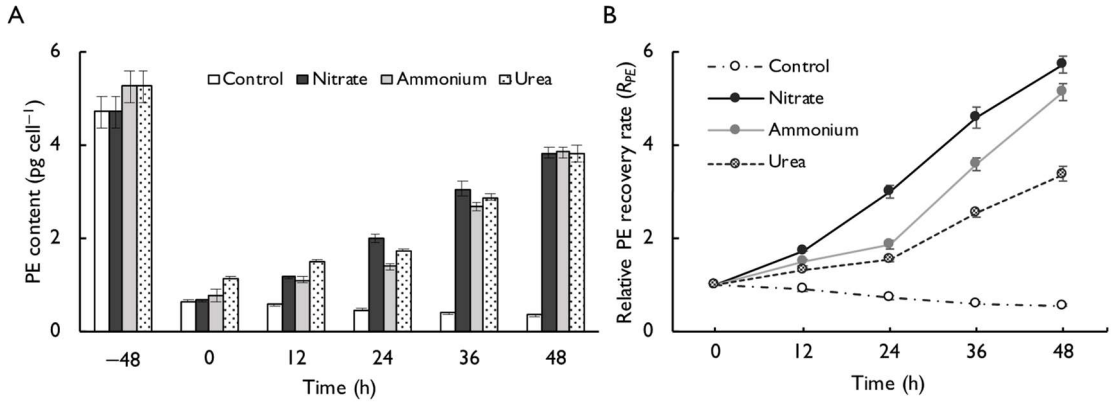
Nitrogen sources, i.e., nitrate, ammonium, and urea, were added to the medium at 0 h. The media for the control group was not supplemented with a nitrogen source. Data are expressed as the mean of three replicates ± SE.

The changes in PE content and relative PE recovery rate (R_{PE}) in *Rhodomonas* sp. are shown in Fig. 2. After the N removal by the medium replacement (at −48 h in the figures), the PE content decreased, and the cells drastically became discolored to greenish as previously reported. The respective PE contents at 0 h were 0.64 ± 0.02 pg cell^{-1} in the control group, 0.67 ± 0.03 pg cell^{-1} in the nitrate group, 0.74 ± 0.13 pg cell^{-1} in the ammonium group, and 1.13 ± 0.02 pg cell^{-1} in the urea group (Fig. 2A). After the supplementation of N sources, the N-supplemented groups exhibited a remarkable increase in PE content, reaching maximum values of 3.8 ± 0.01 pg cell^{-1} in the nitrate group, 3.8 ± 0.12 pg cell^{-1} in the ammonium group, and 3.8 ± 0.18 pg cell^{-1} in the urea group. The respective R_{PE} at 48 h was 0.57 ± 0.02-fold in the control group, 5.73 ± 0.18-fold in the nitrate group, 5.13 ± 0.16-fold in the ammonium group, and 3.36 ± 0.16-fold in the urea group (Fig. 2B). The PE productivity (P_{PE}) for each period is presented in Table 2. The P_{PE} at 0–48 h was −0.02 ± 0.0 μg mL^{-1} h^{-1} in the control group, 0.32 ± 0.01 μg mL^{-1} h^{-1} in the nitrate group, 0.26 ± 0.02 μg mL^{-1} h^{-1} in the ammonium group, and 0.25 ± 0.01 μg mL^{-1} h^{-1} in the urea group, and no significant differences were observed among the three N sources. At 12–24 h, the nitrate group showed a significantly higher

228 P_{PE} of 0.41 ± 0.03 μg mL^{-1} h^{-1} compared to the other groups. In contrast, the highest P_{PE}

229 of 0.45 ± 0.03 μg mL^{-1} h^{-1} in this study was obtained in the ammonium group at 24–36

230 h. In the control group, P_{PE} gave negative values throughout the experiment.



231 **Fig. 2. Phycoerythrin content (A) and relative PE recovery rate (R_{PE}) (B) of**

232 ***Rhodomonas* sp. grown in different nitrogen sources.**

233 Nitrogen was removed from the medium at −48 h. Nitrogen sources, nitrate, ammonium,

234 and urea were respectively supplemented to the medium at time 0 h. The control group

235 was medium without a supplemented nitrogen source. Data are expressed as the mean of

236 three replicates ± SE.

Table 2 Specific growth rate (μ) and PE productivity (P_{PE}) of *Rhodomonas* sp. grown in different nitrogen sources.

Nitrogen source	Specific growth rate (μ, day^{-1})	PE productivity (P_{PE}, μg ml^{-1} h^{-1})				
		0–12 h	12–24 h	24–36 h	36–48 h	0–48 h
Control	−0.02 ± 0.03[b]	−0.02 ± 0.00[b]	−0.02 ± 0.00[c]	−0.02 ± 0.00[b]	−0.01 ± 0.00[b]	−0.02 ± 0.00[b]
Nitrate	0.30 ± 0.02[a]	0.14 ± 0.02[a]	0.41 ± 0.03[a]	0.42 ± 0.04[a]	0.32 ± 0.05[a]	0.32 ± 0.01[a]
Ammonium	0.23 ± 0.03[a]	0.08 ± 0.04[a]	0.16 ± 0.01[b]	0.45 ± 0.03[a]	0.36 ± 0.03[a]	0.26 ± 0.02[a]
Urea	0.26 ± 0.03[a]	0.08 ± 0.00[a]	0.22 ± 0.01[b]	0.35 ± 0.01[a]	0.35 ± 0.02[a]	0.25 ± 0.01[a]

The data represent the mean ± SE (n = 3). Significant differences in the same column are indicated using the same letter ($P < 0.05$)

237

3.2 Gene expression of N transporters

The relative expression of the N transporter genes in *Rhodomonas* sp. grown under N-sufficient and N-starved conditions is shown in Fig. 3. All transporter genes were upregulated under N-starved conditions. The relative expression of *RhNrt2*, *RhAmt1*, and *RhDur3* under N-starved conditions was approximately 16.5-fold, 1.92-fold, and 2.21-fold higher than that under N-sufficient conditions. Significant differences between the two groups were observed for *RhNrt2* and *RhDur3*.

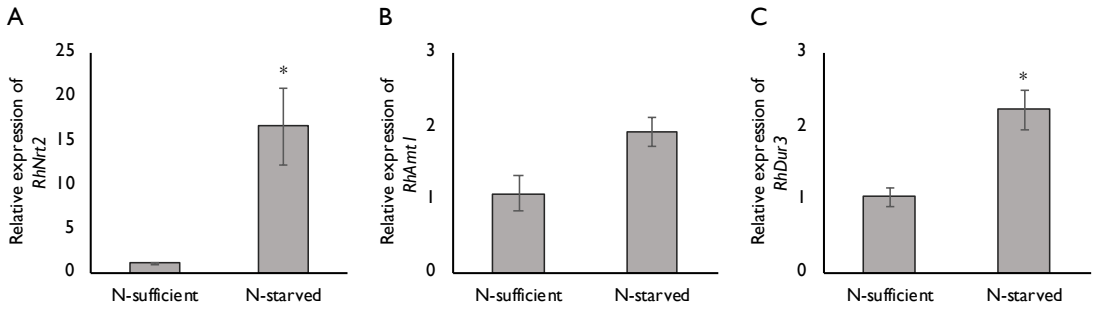


Fig. 3. Relative expression of nitrogen transporter genes, i.e., *RhNrt2* (A), *RhAmt1* (B), and *RhDur3* (C), of *Rhodomonas* sp. grown under nitrogen-sufficient and -starved conditions.

Data are expressed as the mean of three replicates ± SE. Asterisks (*) indicate significant differences ($P < 0.05$).

3.3 Gene expression of cpeb

The relative *RhCpeb* expression patterns of *Rhodomonas* sp. grown using different N sources are shown in Fig. 4. *RhCpeb* was significantly upregulated in all the N-supplemented groups, whereas it was significantly suppressed in the control group and was barely detectable until the end. Relative *RhCpeb* expression surged immediately after nitrate supplementation in the nitrate group and was significantly different from the initial value at 0 h, unlike in the other groups. The maximum *RhCpeb* expression levels were 35.9-fold in the nitrate group, 14.7-fold in the ammonium group, and 18.3-fold in the urea group. In contrast, a decrease in *RhCpeb* expression was observed from 36 to 48 h in the ammonium group.

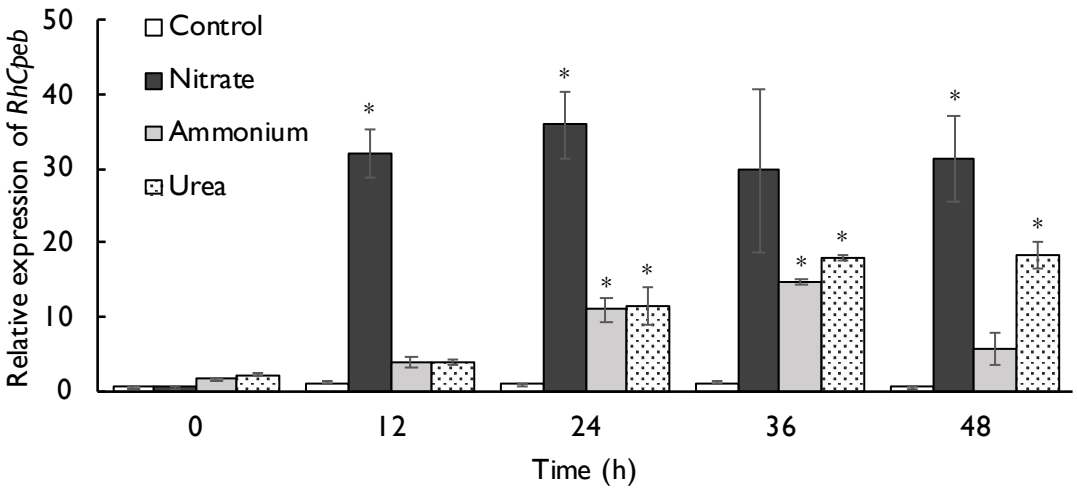


Fig. 4. Changes in the relative expression of *RhCpeb* of *Rhodomonas* sp. grown in different nitrogen sources.

Nitrogen sources nitrate, ammonium, and urea were added to the medium at 0 h. The control group was medium without a supplemented nitrogen source. Data are expressed as the mean of three replicates ± SE. Asterisks (*) indicate significant differences between the initial value at 0 h and each value ($P < 0.05$).

4. Discussion

N is a key biological molecule that profoundly affects cell growth and biosynthesis of bioactive compounds in microalgae. The selection of N sources is directly linked to N availability and influences the biosynthesis of diverse bioactive compounds such as amino acids, lipids, and carotenoids [33–35]. The amounts of intracellular PE has a significant impact on the potential applications of *Rhodomonas* species as feed, given its close association with nutritional value and culture stability. Therefore, selecting the optimal N source is a crucial research avenue for enhancing the overall utility of *Rhodomonas* species as feed in aquaculture. In the present study, we set up a 48 h-N starvation starter culture prior to a batch culture experiment to reliably assess the effect of different N sources on PE recovery in discolored *Rhodomonas* sp. Our previous findings indicated that although *Rhodomonas* sp. accumulates intracellular N compounds and their downstream metabolites, their influence on physiological activities, such as cell growth and biosynthesis of bioactive compounds, tends to diminish approximately 48 h after the transition to N-starved conditions [29]. Consequently, the chosen experimental design was deemed pertinent for scrutinizing the distinct effects of individual N sources.

We also confirmed the capacity of each of the tested N sources in *Rhodomonas* sp. through expression analysis of N transporter genes. The transcript levels of *RhNrt2*, *RhAmt1*, and *RhDur3* were measured after 72 h of N starvation. As shown in Fig. 4, all N transporters were upregulated under N starvation, which aligns with the typical response observed in other microalgae species [36,37]. This supports the conclusion that this species can directly take up and utilize each of the tested N sources.

Although the influence of N sources on PE biosynthesis in cryptophytes remains understudied, extensive research on bacteria has highlighted that the optimal N source for phycobiliprotein biosynthesis is diverse and depends on the species or strain. For example, De Lorimier et al. [38] observed a 10–20% increase in phycocyanin content in *Synechococcus* sp. PCC 7002 when cultured on ammonium rather than nitrate. In contrast, a study by Liotenberg et al. [39] reported a 35% lower PE content in *Calothrix* sp. grown in ammonium than in nitrate. Additionally, Khazi et al. [40] conducted a comparative study on the impact of nitrate and ammonium on phycocyanin biosynthesis in three cyanobacteria: *Phormidium* sp., *Pseudoscillatiria* sp., and *Arthorospira platensis*. Their findings revealed that the former two species exhibited 5–15% higher phycocyanin

298 production when grown in ammonium, whereas nitrate was favorable for the latter species
299 because growth inhibition occurred with ammonium. Our data demonstrated the
300 effectiveness of all tested N sources, including nitrate, ammonium, and urea, for cell
301 growth and PE biosynthesis in discolored *Rhodomonas* sp. In the present study, N
302 starvation treatment for 48 h decreased the intracellular PE content and discolored cells
303 to greenish, but when each N source was supplemented to the medium, the PE content
304 rebounded to approximately 80% of the initial level within 48 h, visually restoring the
305 cell color to bright red. The nitrate group showed the highest specific growth rate and had
306 a more than 10% higher R_{PE} than the other N sources and demonstrated superior
307 immediacy in PE recovery. Therefore, based on these findings, it is considered that among
308 the three N sources tested in this study, nitrate is the most effective N source for PE
309 recovery. To the best of our knowledge, this is the first documented evidence
310 demonstrating the efficacy of N supplementation for PE recovery from discolored
311 *Rhodomonas* species.
312 Mercier et al. [15] proposed that cells undergoing active growth would exhibit lower
313 concentrations of phycobiliproteins because their synthesis may compete with the

314 production of other essential proteins, and cells would preferentially use the metabolic
315 capacity for these vital components. Furthermore, considering that cell division divides
316 the accumulated PE, their explanation positing a conflict between cell growth and
317 intracellular PE content appears plausible. Other studies support this phenomenon; for
318 instance, a comprehensive study by Chaloub et al. [21] on the combined effects of
319 temperature and light on PE biosynthesis revealed a clear negative correlation between
320 specific growth rate and PE biosynthesis. Similarly, a study by Latsos et al. [24] proposed
321 a strong negative correlation between PE content and cell growth, with a coefficient of
322 determination exceeding 0.8. In our current study, despite the active cell growth, the
323 nitrate group showed significantly high PE productivity of 0.41 ± 0.03 μg mL^{-1} h^{-1} in the
324 12–24 h period (Table 2), comparable with values reported in previous studies mentioned
325 above. This result suggests that there was no critical competition between cell growth and
326 PE biosynthesis in the present experimental setup. The compatibility observed between
327 PE biosynthesis and cell growth can be attributed to the high N concentration (7.0 mM
328 used in this study), which exceeded the species growth requirements. We previously
329 reported that the maximum N requirement for this species was 3.5 mM and that no

330 positive effect on growth occurred beyond this concentration [28]. On the other hand,
331 Madkour et al. [41] reported that, irrespective of the N requirement for growth, increasing
332 the N concentration in the medium boosts the biosynthesis of proteins, including
333 photosynthetic pigments. Considering these reports and the present results, the
334 application of a high N concentration would be effective for recovery of discolored
335 *Rhodomonas* species, irrespective of their N requirements. However, caution should be
336 exercised as the use of excessive nitrogen sources in aquacultural sites can lead to
337 eutrophication of the natural marine environment via effluents. In addition, the use of
338 high concentration of ammonium may be inappropriate as it may induce acidification of
339 the medium due to excess protons released during the ammonium assimilation process,
340 leading to cell lysis [27,42]. Indeed, in the present study, when incubation was extended
341 beyond 48 h, cell mortality, possibly due to medium acidification, was observed only in
342 the ammonium group at 72 h (data not shown). In the future, regardless of the N source
343 used, it will be necessary to investigate the appropriate N supply for PE recovery, taking
344 into account the above issue.
345 Our previous work [29] proposed that *RhCpeb,* encoding the β-subunit of PE, serves as a

346 good molecular indicator of PE biosynthesis in *Rhodomonas* sp., with its expression
347 pattern closely reflecting PE biosynthesis dynamics. In the present study, we expected a
348 reappearance of *RhCpeb* expression, which diminished with N starvation upon N
349 supplementation. The obtained results support this prediction, revealing that all three N
350 sources tested significantly re-promoted *RhCpeb* expression compared to the control
351 group, which exhibited no increase in expression. The distinct expression profiles
352 observed for each N source are noteworthy. The ammonium and urea groups exhibited a
353 gradual increase in expression, whereas the nitrate group consistently showed strong
354 expression immediately after N source supplementation. Interestingly, these expression
355 patterns in each group broadly mirrored the trends observed in the PE productivity results.
356 Phycobiliprotein biosynthesis involve intricate regulatory mechanisms. In their study
357 investigating the impact of light spectra on pigment concentration and the expression of
358 photosynthesis-related genes in *R. salina*, Schomaker et al. [43] suggested that the
359 transcriptional levels of related genes, such as *RhCpeb* and *RhCpea* (encoding the *α*-
360 subunit of PE), did not necessarily reflect the actual pigment concentrations. They
361 attributed this disparity to the regulation of phycobiliprotein biosynthesis, which is

362 governed by post-transcriptional or post-translational modifications. Although this study
363 did not delve into such epigenetic dynamics in PE biosynthesis, our findings suggest a
364 potential correlation between intracellular N metabolites and the regulation of
365 phycoerythrin synthesis. Analysis of variations in *RhCpeb* expression profiles for each N
366 source obtained in this study could offer valuable insights into the complex regulatory
367 mechanisms that dictate intracellular PE biosynthesis in cryptophytes.

368 **5. Conclusion**

369 We investigated the effects of three different N sources, i.e., nitrate, ammonium, and urea,
370 on the recovery of PE content in discolored *Rhodomonas* sp. Our investigation
371 highlighted the efficiency of all three N sources in promoting PE recovery, with nitrate
372 exhibiting the highest performance in PE biosynthesis. These findings were further
373 validated by expression analysis using *RhCpeb* as a molecular indicator, emphasizing the
374 potential of nitrate as the most favorable N source for *Rhodomonas* species. This study is
375 the first to demonstrate the recoverability of PE content in discolored *Rhodomonas* sp.
376 through supplementation with an appropriate N source, even after complete cell
377 discoloration. These findings pave the way for informed strategies for the stable

management and production of *Rhodomonas* species as feed for aquaculture.

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Data availability

The biological materials and data generated in this study are available upon request from the corresponding authors.

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