Iranian Journal of Fisheries Sciences 20(2) 358-369 2021
DOI: 10.22092/ijfs.2021.123786

Research Article

Effeciency of *Spirulina platensis* cultured with different nitrogen regimes on larval development, growth and survival rate of white shrimp, *Litopenaeus vannamei* n.R.[1]

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Received: August 2019 Accepted: May 2020

Abstract

The blue-green algae *Spirulina platensis* was cultured with different nitrogen regimes (NH_4Cl, NH_4NO_3 and KNO_3) with concentrations of 0.010, 0.025 and 0.050 M and then fed to Litopenaeus *vannamei* at 19 days post hatch (PL_I) to evaluate the nutritional quality. At the end of the experiment, growth and survival rate were determined, and the larval development was calculated. The maximum protein content was obtained in culture media containing NH_4NO_3 followed by NH_4Cl and KNO_3 ($p>0.05$). Moreover, in all *S. platensis* cultures, increases in nitrogen concentrations led to increases in protein content. The present study showed that, with the exception of shrimp larvae at 5 days post hatch, *L. vannamei* shrimp larvae fed on *S. platensis* cultured in NH_4NO_3, as a nitrogen regime, generally developed significantly ($p<0.05$) faster to next stages compared with the other treatments. However, the survival rate (%) and total length (mm) of shrimp larvae fed on *S. platensis* did not show any significant difference ($p>0.05$) when the nitrogen was varied in terms of regimes. Overall, the study points to the potential effectiveness of using NH_4NO_3 as a nitrogen regime in *S. platensis* culture media in producing live food for the hatchery production of *L. vannamei* shrimp larvae.

Keywords: Nitrogen, *Spirulina platensis*, Survival rate, Larval development, *Litopenaeus vannamei*

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Introduction

The cost and composition of culture medium to biomass production of the microalgae are challenging factors for commercially viable production (Ilavarasi *et al.,* 2011). The first synthetic medium formulated for cultivation of *Spirulina* was Zarrouk's medium (Zarrouk, 1966) which is still used as the standard medium. Subsequently, different media have been tried for cultivation of *Spirulina* such as Rao's media (Singh, 2006), CFTIR media (Venkataraman *et al.,* 1995) and OFERR media (Singh, 2006) The production of *Spirulina* biomass requires water soluble forms of phosphorus and nitrogen (Mostert and Grobbelaar, 1987), and also sources of nutrition affect the growth rate and biochemical composition of *Spirulina*. The conventional nitrogen source for *S. platensis* is nitrate salts (sodium and potassium nitrates), and several studies have demonstrated the feasibility of replacing these conventional nitrogen sources with low-cost alternatives such as urea (Matsudo *et al.,* 2009; Avila-Leon *et al.,* 2012), ammonium sulfate (Ferreira *et al.,* 2010) and ammonium chloride (Bezerra *et al.,* 2008).

In the early stages of larval shrimp production, the supply of live food such as microalgae with high nutritional quality is one of the crucial activities in hatcheries (Spolaore *et al.,* 2006). Therefore, shrimp hatcheries are highly dependent on microalgae which are costly and labor intensive. Finding the efficient feeds for white shrimp larvae is necessary in order to increase post-larvae quality and culture profitability. The dependence of commercial hatcheries on *Artemia* nauplii production will be a concern due to the possibility of rising cost of *Artemia* cyst, as well as the possible risk of disease transfer. Moreover, up to 80% of hatcheries have decreased using *Artemia* cyst in the last few years as a result of these concerns ((Lavens, and Sorgeloos, 2000)). In Iran, commercial hatcheries of *Litopenaeus vannamei* post larvae production need live food such as microalgae and *Artemia* nauplii as food at larval stages in their life cycle.

The major nutritional interest in *Spirulina* is due to high protein content, ease of digestion and significant contents of vitamins, minerals, amino acids and pigments (Piñero Estrada *et al.,* 2001; Marrez *et al.,* 2014; Kermani *et al*., 2020). The cyanobacterium *Arthrospira* (*Spirulina*) *platensis* has even been used by humans because of its nutritional and possible medicinal effects (Colla *et al*., 2007). Due to the fact that, *Spirulina* has many biological active substance, it has been used in aquaculture (Gouveia *et al.,* 2008) as well as poultry (Carrillo *et al.,* 2008) and ruminant (Kuplys *et al.,* 2009) nutrition. To date, a number of studies have been conducted using dried *Spirulina* as a feed supplement (Jin *et al*., 2020; Holman and Malau-Aduli, 2012), while no study was conducted to use it in *L. vannamei* post larval production in Iran. Therefore, the main

objective of this study was to evaluate the performance of *L. vannamei* larvae (body length, survival and development index) fed on *S. platensis* cultured with different nitrogen regimes.

Materials and methods

Microalgae culture

S. platensis was grown in modified Zarrouk's medium (Rajasekaran *et al.*, 2016) in a 3000-ml Erlenmeyer flask, in which the $NaNO_3$ was replaced by NH_4Cl, NH_4NO_3 and KNO_3 with concentrations of 0.010, 0.025 and 0.050 M. Culture mediums were incubated at 30°C, salinity of 25 ppt and initial pH of 9.5 under 12:12 hour light-dark photoperiod with normal white light (Guillard and Ryther, 1962).

Protein analysis

The *S. platensis* samples were harvested during the exponential growth phase (day 8) to determine protein content. Cells were concentrated using a centrifuge (Universal Tlettich, Germany) at 5000 g for 10 min and then freeze-dried (ZirBus, VaCo 5) before being stored at -25°C until biochemical analysis. Total protein was determined by the method of Lowry *et al.* (1951).

Larvae culture and feeding trial

Newly hatched *L. vannamei* (nauplii 5; N_v) larvae were supplied by a private shrimp hatchery (Sontderaf, Bandar Jask, Iran) where this study was conducted. For acclimation to the experimental conditions, the shrimp nauplii were briefly kept in a 20-L tank filled with filtered and UV-treated seawater at 30°C and 35 mg L^{-1} salinity. At the start of the feeding trial, the shrimp nauplii were stocked in 5-L Erlenmeyer flasks filled with 3 L of filtered seawater at a density of 100 nauplii L^{-1} and reared for 19 days. The water was exchanged at a rate of 60–70% every other day. Three separate feeding regimes (consist of three replicates each) were conducted concurrently to evaluate their nutritional quality on early larval development, growth and survival of *L. vannamei* from nauplii 5 (N_v) to postlarvae 9 (PL_{IX}). The feeding regimes were as follows:

Larvae fed *S. platensis* that had been cultured with:

(1) NH_4Cl (Diet A);

(2) NH_4NO_3 (Diet B); and

(3) KNO_3 (Diet C).

Larval development, total length and survival rate

The early larval development, survival rate and total length of nauplii were evaluated at 5, 9, 14 and 19 days post hatch. The larval developmental stages were determined according to criteria defined by Ronquillo *et al.* (2006) for the larvae of *Penaeus semisulcatus*. The total length of individual shrimp larvae was measured to the nearest 0.01 mm by random sampling of 10 larvae from each dietary regime and under a binocular microscope from the tip of the rostrum to the end of the telson using an electronic digital caliper. The

mean survival rate (%) was calculated on given days by counting all survived larvae using glass pipettes, dividing by the total number that was initially stocked (300), and multiplying by 100.

Statistical analysis

Data were analyzed with SPSS 16.0 software using parametric tests and statistical analysis. A one-way ANOVA was used to determine significant differences. Duncan's multiple range tests (Duncan, 1955) were used to rank the treatments and mean differences were considered significant at $p<0.05$.

Results

Protein analysis

Figure 1 shows the protein content of microalga *S. platensis* following culture with different nitrogen regimes (NH_4Cl, NH_4NO_3 and KNO_3). The protein content was slightly higher in media containing NH_4NO_3 than in that containing NH_4Cl and KNO_3. However, in all *S. platensis* cultures, increasing in nitrogen concentrations led to an increase in protein content; but there was not significant difference in terms of nitrogen regimes or concentrations ($p>0.05$).

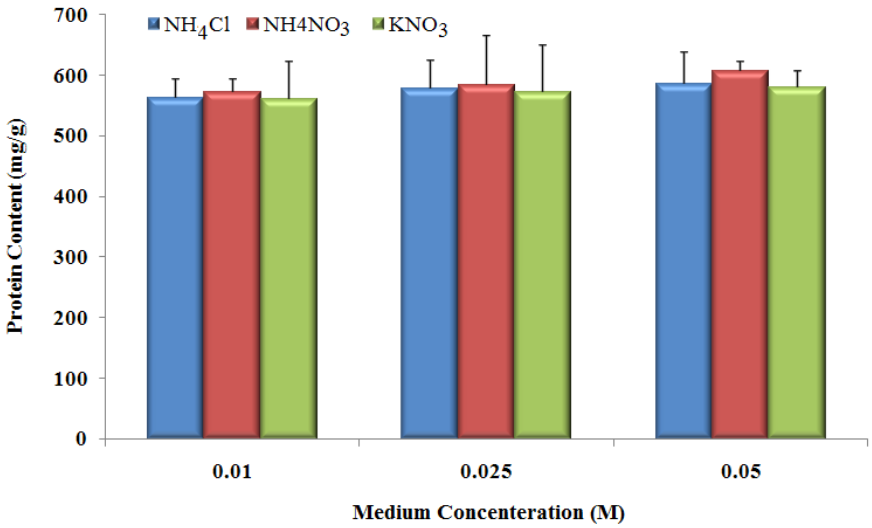

Figure 1: Protein content of microalga *Spirulina platensis* following culture with different nitrogen regimes.

Larval stage development and survival rate

Larval stage development and survival rate of *L. vannamei* fed *S. platensis* cultured with different nitrogen regimes at 5 days post hatch is shown in Table 1. The result showed that *L. vannamei* fed with different *S. platensis* cultured with different nitrogen regimes could successfully develop to protozoa 3 (PZ_{III}) at 5 days post hatch and in this case there was no significant difference in terms of nitrogen regimes ($p>0.05$). Moreover, no notable differences were found in terms of survival rate in different treatments.

Table 1: Larval development and survival rate of *Litopenaeus vannamei* fed on *Spirulina platensis* cultured with different nitrogen regimes at 5 days post hatch*.

Treatments**	Stage composition (%)†				Survival Rate (%)
	N_V	PZ_I	PZ_{II}	PZ_{III}	
Diet A	3±0	9±1	9±1	79±5	83±7
Diet B	3±0	6±0	10±2	81±7	84±9
Diet C	3±0	8±1	9±0	80±11	82±6

*Data are mean values of triplicate samples ± SD.; Different superscript letters in the same column indicate significant differences (p<0.05).

** NH_4Cl (Diet A); NH_4NO_3 (Diet B); KNO_3 (Diet C).

†Stage composition; Nv= Naplius 5; PZ_I = protozoa 1, PZ_{II} = protozoa 2 and PZ_{III} = protozoa 3 as % of total live larvae.

The results of larval stage development and survival rate of *L. vannamei* on the 9th day after hatching that were fed on *S. platensis* cultured with different nitrogen regimes, is shown in Table 2. According to the results, shrimp larvae fed on diets B and C developed slightly faster to PL_I stage compared to larvae fed on diet A. On the other hand, there was no significant difference in terms of survival rate in different treatments at 9 days post hatch (p>0.05).

Table 2: Larval stage composition and survival rate of *Litopenaeus vannamei* fed on *S. platensis* cultured with different nitrogen regimes at 9 days post hatch*.

Treatments	Stage composition (%)†				Survival Rate (%)
	M_I	M_{II}	M_{III}	PL_I	
Diet A	5±1	7±1	15±2	73±8	65±5
Diet B	6±0	5±0	11±1	78±6	69±7
Diet C	3±1	7±2	13±4	77±6	64±8

*Data are mean values of triplicate samples ± SD.; Different superscript letters in the same column indicate significant differences (p<0.05).

** NH_4Cl (Diet A); NH_4NO_3 (Diet B); KNO_3 (Diet C).

†Stage composition; M_I = Mysis 1, M_{II} = Mysis 2, M_{III} = Mysis 3 and PL_I = Post larvae 1 as % of total live larvae.

On the other hand, *L. vannamei* larvae that were fed on *S. platensis* cultured with NH_4NO_3 developed significantly (p<0.05) faster to PL_V stage compared with the other diets on the 14th day after hatching (Table 3). On the same day, survival rate of *P. vannamei* larvae was significantly higher when fed on NH_4NO_3 compared with the other diets (p<0.05).

Upon termination of the experimental period (19th day after hatching), most shrimp larvae successfully molted to PL_{IX} (77% for Diet B, 72% for Diet C, and 70% for Diet A). A significantly higher percentage of shrimp larvae, which developed to PL_{IX} stage and fed on *S. platensis* that was previously cultured with NH_4NO_3 as a nitrogen regime in culture media (p<0.05) was found. Moreover, the survival rate was not significantly different in tested dietary regimes, following feeding on *S. Platensis* (Table 4).

Table 3: Larval stage composition and survival rate of *Litopenaeus vannamei* fed on *S. platensis* cultured with different nitrogen regimes at 14 days post hatch*.

Treatments	Stage composition (%)†				Survival Rate (%)
	PL_{II}	PL_{III}	PL_{IV}	PL_{V}	
Diet A	6±2	10±2	14±2	$70±9^{b}$	$48±6^{b}$
Diet B	4±1	8±2	11±4	$77±10^{a}$	$57±9^{a}$
Diet C	4±1	11±1	13±2	$72±6^{b}$	$51±5^{b}$

*Data are mean values of triplicate samples ± SD.; Different superscript letters in the same column indicate significant differences ($p<0.05$).

** NH_4Cl (Diet A); NH_4NO_3 (Diet B); KNO_3 (Diet C).

†Stage composition; PL_{II} = Post larvae 2, PL_{III} = Post larvae3, PL_{IV} = Post larvae 4 and PL_{V} = Post larvae 5 as % of total live larvae.

Table 4: Larval stage composition and survival rate of *Litopenaeus vannamei* fed on *S. platensis* cultured with different nitrogen regimes at 19 days post hatch*

Treatments	Stage composition (%)†				Survival Rate (%)
	PL_{VI}	PL_{VII}	PL_{VIII}	PL_{IX}	
Diet A	2±0	8±2	11±3	$79±6^{b}$	35±5
Diet B	1±1	6±1	8±2	$85±12^{a}$	41±2
Diet C	3±1	6±0	10±2	$81±9^{b}$	39±7

*Data are mean values of triplicate samples ± SD.; Different superscript letters in the same column indicate significant differences ($p<0.05$).

** NH_4Cl (Diet A); NH_4NO_3 (Diet B); KNO_3 (Diet C).

†Stage composition; PL_{VI} = Post larvae 6, PL_{VII} = Post larvae 7, PL_{VIII} = Post larvae 8 and PL_{IX} = Post larvae 9 as % of total live larvae.

Total length

The mean final length of *L. vannamei* larvae (on the 19th day of the feeding trial) fed on *S. platensis* that were previously cultured with different nitrogen regimes is shown in figure 2. Although the maximum final length (Diet B; 8600µ) was obtained in culture containing NH_4NO_3; but there was no significant differences between treatments for larval size when the nitrogen were varied in term of regimes ($p>0.05$).

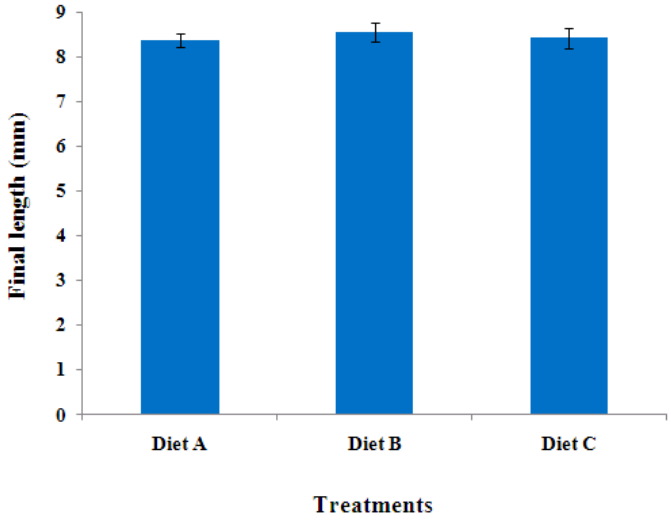

Figure 2: Final length (mm) of *Litopenaeus vannamei* larvae following culture with different nitrogen regimes.

Discussion

Providing a suitable nitrogen regime in culture medim can be considered as a promising method to *S. platensis* cultivation for achieving optimal biomass and protein production (Salunke *et al.,* 2016). Moreover, cultivation medium has a great impact on the productivity of biomass and other compounds of interest. For example, nitrogen concentration in the medium (optimum at 2.5 g/L) and also nitrogen source (urea better than ammonium or nitrate) has a great impact on *Spirulina* productivity (Soletto *et al.,* 2005; Çelekli and Yavuzatmaca, 2009). Therefore, in this investigation, different nitrogen regimes were chosen to evaluate those effects on the biomass and protein content of microalga *Spirulina platensis*. We determined that the protein content of *S. platensis* cultured with NH_4NO_3 was slightly higher than cultures containing NH_4Cl or KNO_3. Moreover, in all *S. platensis* cultures, increasing nitrogen concentrations led to a slight increase in protein content, but it was not significantly different in terms of nitrogen regimes. The reason for the slightly higher protein amount in cultures with NH_4NO_3 than cultures containing NH_4Cl or KNO_3 can be explained as the NH_4NO_3 contains two nitrogen atoms (35% nitrogen) than to NH_4Cl or KNO_3 which have only one nitrogen atom (22 or 14% nitrogen, respectively) (Faintuch *et al.,* 1991). Moreover, the reason for the slightly lower protein amount in cultures with low nitrogen concentrations can be interpreted as the depletion of the nitrogen in the culture medium as a result of faster growth and the prolonged steady state. Uslu *et al.* (2011) mentioned that in N-sufficient growth mediums, protein content of *S. platensis* is supported, while its content drops in N-deficient mediums.

Spirulina is considered as one of the most concentrated natural sources for nutrition to both terrestrial and aquatic animals. Therefore, *Spirulina* could be an excellent source of useful nutrients as well as a good energy source that can be used as a crucial component for animal feeding (Farag *et al.,* 2016). However, the main application of microalgae for aquaculture is associated with nutrition and have beneficial effects on stabilizing water quality and sbsequently the health of aquatic animals (Han *et al.,* 2019). In this study, the nutritional effect of *Spirulina* cultured with different nitrogen regimes was further evaluated using *L. vannamei* larvae.

The larval development showed that *L. vannamei* fed with *S. platensis* cultured with different nitrogen regimes could successfully develop to next stages, and there was no significant difference before 9 days post hatch larvae (PL_I). However, in general, it should be noted that *L. vannamei* larvae fed on *Spirulina* that were previously cultured with NH_4NO_3 as a nitrogen regime, moulted faster to next stages compared with larvae fed on cultures containing NH_4Cl and KNO_3,

in particular beyond the 14th day after hatching. Delayed or slightly lower larval development rates observed in cultures containing NH_4Cl or KNO_3 could be due to inadequate nutrients or energy from *S. platensis* for *L. vannamei* larvae, which likely hindered development to the next stages. It is well known that there is a relationship between larval development rates for penaied shrimp and protein requirement (Cuzon, 2004); and shrimp larvae have a higher dietary protein requirement than juveniles and adults (Lee and Lee, 2018). Moreover, larval development of *L. vannamei* could only be supported with a diet of *S. platensis* until the M_{III}/PL stage, and beyond this stage, the larvae need *Artemia* nauplii as live feed. This is in agreement with the report by Ronquillo *et al.* (1997) on the larval development with other penaeid larvae of *P. semisulcatus*, *P. monodon* and *P. chinensis*.

On the same way, higher larval survival rates were obtained in *L. vannamei* larvae fed on *Spirulina* that were previously cultured with NH_4NO_3 as a nitrogen regime. *L. vannamei* larvae fed on this diet clearly showed significant increases in survival rate beyond the 14th day after hatching, and before this stage (PL_I), in general, shrimp larvae in all tested diets exhibited equal survival rates on the feeding regimes. This result indicated that the tested diets could provide nutritional requirements and subsequently promote higher survival rates for *L. vannamei* larvae. Colvin and Brand (2009) indicated that the optimum level of dietary protein requirement by penaeid shrimp varies in different larval stages, which corresponds to the results of this study.

Indeed, similar total length of shrimp larvae was observed with the feeding trials. Jaime-Ceballos *et al.* (2005) stated that supplementation of *S. platensis* meal did not favor an increment in size of *Litopenaeus schmitti* larvae. Moreover, Teshima *et al.* (1982) indicated that growth measured as final total length may not be the best criteria for evaluating larval feeding experiments; instead, success of metamorphosis is suggested as a better indicator of the nutritional value of a larval diet. Nevertheless, the final total length of shrimp larvae observed in this study was in agreement with the larval development rate since we monitored both parameters (total length and larval development). In conclusion, this study clearly showed that using NH_4NO_3 as a nitrogen regime in *S. platensis* culture media could provide a better diet to meet nutritional requirements and subsequently promote higher survival, growth rate and larval development for the hatchery production of *L. vannamei* larvae.

Acknowledgments

The authors thank the Persian Gulf and Oman Sea Ecology Research Center for their technical support. The authors are grateful to Dr. Samuel Allen for his editorial assistance.

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