Harmful Algae 8 (2009) 239–246

Contents lists available at ScienceDirect

Harmful Algae

journal homepage: www.elsevier.com/locate/hal

Macronutrients requirements of the dinoflagellate *Protoceratium reticulatum*

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ARTICLE INFO

Article history:
Received 24 January 2008
Received in revised form 4 June 2008
Accepted 4 June 2008

Keywords:
Dinoflagellates
Gonyaulax grindleyi
Nitrate
Phosphate
Protoceratium reticulatum
Yessotoxins

ABSTRACT

The basal L1 medium was found to be unsatisfactory for culturing the red tide dinoflagellate *Protoceratium reticulatum* at a high growth rate and biomass yield. The L1 medium enhanced with phosphate to a total concentration of 217 μM supported the highest attainable growth rate and biomass yield. Once the phosphate concentration exceeded 6× L1, phosphate inhibited the dinoflagellate growth and negatively affected cell viability. At the optimal phosphate concentration of 217 μM, an increase in nitrate concentration over the range of 882–8824 μM, did not affect cell growth and yield. Nitrate did not inhibit growth at any of the concentrations used. Clearly, the basal nitrate level in L1 is sufficient for effectively culturing *P. reticulatum*. At the ranges of phosphate and nitrate concentrations tested, cell volume was not sensitive to the concentration of nutrients but the concentration of phosphate affected both the specific cell number and cell volume growth rates. Elevated levels of nutrients supported their intracellular accumulation. Cell-specific production of yessotoxin was not influenced by concentration of phosphate in the culture medium, but elevated (>1764 μM) nitrate concentration did enhance the yessotoxin level. Phosphate concentration that maximized biomass yield also maximized volumetric production of yessotoxin in the culture broth.

1. Introduction

Blooms of toxic dinoflagellates are associated with fish kills, poisonings of marine wildlife, and seafood poisonings of humans (García Camacho et al., 2007b). Dinoflagellates are very shear-sensitive micro-organisms (García Camacho et al., 2007a) that produce various toxins (García Camacho et al., 2007b). Some of these toxins are of potential interest in human medicine and quantities of pure toxins are also needed as analytical standards that are used in identifying and quantifying toxins in natural samples. Production of dinoflagellate toxins for various purposes requires an understanding of the nutritional requirements for dinoflagellate growth and toxin production (Kobayashi and Kubota, 2007; García Camacho et al., 2007b). The dinoflagellate *Protoceratium reticulatum*, also known as *Gonyaulax grindleyi*, is a producer of yessotoxins (YTXs). *P. reticulatum* is widespread in the world's oceans (Ciminiello et al., 2003; Satake et al., 1997, 1999; Paz et al., 2007) and is of particular concern in shellfish aquaculture (Guerrini et al., 2007). YTXs are disulfated polyether compounds that are potent cytotoxins. Yessotoxins are further reviewed by Bowden (2006).

Environmental conditions are believed to be important for toxin expression in dinoflagellates and other microalgae. For example, production of paralytic shellfish poisoning (PSP) toxins by *Alexandrium* spp. appears to be higher at low concentrations of phosphorous (Anderson et al., 1990; Beani et al., 2000) and low salinity values (Hwang and Lu, 2000). The effect of environmental factors on the total amount of toxins produced has been found to be highly dependent on algal species (Guerrini et al., 2007).

This work reports on the production of yessotoxins by *P. reticulatum* as a potential commercial producer for this compound for use in research. Only a few studies have focused on YTX production in this dinoflagellate. Seamer (2001) investigated the effects of nutrients, light, salinity and temperature on YTX production. Mitrovic et al. (2004) evaluated the effects of selenium, iron and cobalt supplementation on growth and YTX production. Paz et al. (2006) examined the influence of temperature, irradiance and salinity on a *P. reticulatum* strain from Spain. Guerrini et al. (2007) reported on the effects of nitrate and phosphate supplementation on growth and YTX production. Toxin production in toxic dinoflagellates has been found to be elevated under low phosphate conditions (Frangópulos et al., 2004; Guerrini et al.,

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doi:10.1016/j.hal.2008.06.002

2007), although most studies agree that nitrogen or phosphorus limitation reduces growth of dinoflagellates (Gallardo Rodríguez et al., 2007; Shi et al., 2005; Siu et al., 1997; Wang and Hsieh, 2002).

2. Materials and methods

2.1. Species and culture medium

P. reticulatum strain GG1AM kindly donated by the Centro Oceanográfico de Vigo, Spain, was used. This strain had been isolated from the Mediterranean Sea and was already adapted to grow in the L1 medium (Guillard and Hargraves, 1993) that was used in all experiments. The L1 medium was prepared in Mediterranean Sea water. The pH was adjusted to 8.7 with 3 M HCl or NaOH. The medium was filter sterilized (0.22 μm Millipore filter; Millipore Corporation, Billerica, MA, USA). The salinity was approximately 35‰. The typical composition of the Mediterranean Sea water has been published (Contreras et al., 1998). The inocula were maintained in thermostated chambers at 17 °C in 2 L static Erlenmeyer flasks. A 12:12 h light/dark cycle was used with an average irradiance on the surface of culture of 50 μE m^{-2} s^{-1}. Cool white fluorescent lamps (30 W) were used for lighting. Every 7 days 40% of the inoculum by volume was replaced with fresh L1 medium.

2.2. Growth experiments

Two series of experiments were carried out, as follows:

(1) In the first series, the initial phosphate concentration in the medium was varied in separate experiments, starting from 36.23 μM, the basal phosphate concentration of the L1 medium. The phosphate concentrations in separate experiments were 1× L1 (i.e. 36.2 μM), 2× L1, 4× L1, 6× L1, 8× L1 and 10× L1 (i.e. 362.3 μM). The initial nitrate concentration was always 882.42 μM, i.e. the basal level in the L1 medium.
(2) In the second series of experiments, the initial nitrate concentration in the medium increased in steps in separate cultures from that of the basal L1 medium (i.e. 882.42 μM) by a factor of 10. In this series, the phosphate concentration was held constant at the level that had been identified in the series 1 experiments to give the best biomass production results.

Experiments were carried out in static fed-batch cultures in 100 mL Erlenmeyer flasks filled with 50 mL of freshly inoculated *P. reticulatum* broth. Initial cell concentration was about 10,000 cells mL^{-1} in all cases. A culture in mid-exponential growth was used for inoculation. For inoculation, the cells were recovered by centrifugation (75 × *g*) and resuspended in fresh medium of appropriate composition that depended on the specific experiment. Flasks were arranged in rows and were illuminated on one side by two white fluorescent lights. All flasks received the same irradiance level of 50 μE m^{-2} s^{-1} on the illuminated side. The same inoculum was used for all flasks in a given series. All assays of macronutrients were carried out in duplicate. At the end of the culture run, a 15-mL sample was taken from each flask for determination of yessotoxins.

Samples for measuring nitrate, phosphate and the cell concentration were taken at the same time (9:00 a.m.) each day after gentle manual mixing of the flasks. Samples were analyzed immediately. After determining the concentrations of macronutrients, highly concentrated aliquots of nitrate and phosphate were added to the cultures (always before 10:30 a.m.) in order to replenish the macronutrients that had been consumed by the cells during the previous day.

For measuring cell growth, a 1-mL sample was fixed with Lugol's solution (Paz et al., 2004) by mixing a 2:100 (v/v) ratio of Lugol's solution and the sample (Gallardo Rodríguez et al., 2007). Cells were counted immediately under a light microscope using a Sedgewick-Rafter chamber.

2.3. Determination of phosphate and nitrate concentrations

Phosphorous species were measured as PO_4^{3-} using the well-known method of the American Public Health Association (APHA, 1995). Nitrate was quantified by HPLC using a UV detector (Gallardo Rodríguez et al., 2007; Moya, 1996).

2.4. Yessotoxins

Yessotoxins were measured separately in the biomass and in culture supernatant following the method of Paz et al. (2004). An HPLC system (Shimadzu AV10; Shimadzu Corporation, Kyoto, Japan) with a fluorescence detector (Shimadzu RF-10AX) and an autoinjector (Shimadzu SIL-10ADVP) was used.

2.5. Cell volume

Mean cell diameter was measured by forward light scatter method using a Coulter Epics® XL-MCL (Beckman Coulter, Inc., Fullerton, CA, USA) flow cytometer. The cytometer was calibrated by measuring the forward scatter of light caused by different-sized latex beads (5, 10, 15, 20, 25 and 30 μm). Cell volume was calculated as $\pi D_e^3/6$ where D_e was the diameter of a sphere that at an equivalent concentration as the cells in suspension, produced the same forward scatter as the suspension of cells (Hillebrand et al., 1999; Paz et al., 2007).

2.6. Cell viability

To quantify the fraction of viable cells, 100 μL of fluorescein diacetate (1 μg mL^{-1}) was added to 1 mL of cell suspension. Esterases in viable cells convert fluorescein diacetate to a fluorescent compound that emits at 505–545 nm. Fraction of viable population was calculated as the ratio of the number of fluorescent events detected at the above wavelength and the total cell count measured by the cytometer.

2.7. Free internal nutrients

To quantify free internal nitrate and phosphate 1 mL of culture was centrifuged (75 × *g*) and the supernatant was discarded. Afterwards, the cell pellet was disaggregated in 1 mL of demi-water and subjected to sonication for 10 min for cell break-up. The suspension was then centrifuged (1000 × *g*) and nutrients were measured in the clear supernatant as described above.

2.8. Kinetic parameters

The specific cell growth rate μ_{cell} (day^{-1}) was calculated using the following equation:

$$\mu_{cell} = \frac{\ln N_{i+1} - \ln N_i}{t_{i+1} - t_i}, \quad (1)$$

where N_{i+1} and N_i are the cell concentrations (cells mL^{-1}) at times t_{i+1} and t_i (day), respectively. The specific cell volume growth rate μ_{vol} (day^{-1}) was calculated using the following equation:

$$\mu_{vol} = \frac{\ln V_{i+1} - \ln V_i}{t_{i+1} - t_i}, \quad (2)$$