



Research Article

A Biomass Upscale System for the Marine Dinoflagellate *Prorocentrum lima* and the Production of Bioactive Lipophilic Toxins

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Abstract

Biotechnological applications of toxic marine dinoflagellates include seafood safety and biomedical, pharmaceutical and research purposes of their toxins among other bioproducts and bioactive compounds that they produce. The supply of sufficient quantities of phycotoxins for investigational uses remains in demand. Some of these toxins, available in small amounts, are quite expensive while their chemical synthesis is complex and costly. However, some quantities of these toxins could be produced with mass cultures of the appropriate dinoflagellates, which are however difficult to handle. *Prorocentrum lima* is a cosmopolitan marine dinoflagellate, which synthesizes toxins that cause a diarrhetic syndrome to humans through the consumption of contaminated shellfish and fish (Diarrhetic Shellfish Poisoning, DSP). The aim of this study was to design and develop a biomass upscale system for the production of the lipophilic toxins okadaic acid (OA) and dinophysistoxin 1 (DTX1) from the produced biomass of *Prorocentrum lima*. In our study, *P. lima* was grown in large scale semi-continuous cultures under controlled laboratory conditions. The maximum biomass produced was 20690 cells/mL. Maximum toxin production was 63.66 ng/mL for OA and 8.07 ng/mL for DTX1. Toxin quota in *P. lima* cells was 88.7% OA and 11.3% DTX1. The produced culture volume was 300 L and the total volume capacity of the upscale system could reach 500 L.

Keywords: biomass upscale, *Prorocentrum lima*, bioproducts, lipophilic toxins, okadaic acid, dinophysistoxin 1.

Introduction

Toxins that are produced by marine dinoflagellates are used for biomedical, pharmaceutical and research purposes apart from the seafood safety control. Lipophilic marine toxins are a large category of phycotoxins or biotoxins produced by marine dinoflagellates, which involves the okadaic acid-group toxins (OA), yessotoxins, pectenotoxins and azaspiracids (Otero and Alfonso, 2015). OA and its

derivatives, e.g. dinophysistoxin 1 (DTX1) and dinophysistoxin 2 (DTX2) etc, are polyether compounds that cause the Diarrhetic Shellfish Poisoning (DSP) syndrome to humans (Quilliam, 2003). They are produced by the microalgae genera *Dinophysis* and *Prorocentrum*. *Prorocentrum lima* (Ehrenberg) Dodge is a cosmopolitan marine Dinoflagellate that synthesizes DSP toxins in its cells, causing shellfish and fish poisonings in many coastal areas (Faust and Gullledge, 2002).

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OA presents a great biotechnological value in medical research and has facilitated the understanding of several cellular processes (Martínez *et al.*, 2015). OA-group toxins are specific inhibitors of protein Phosphatase 1 and Phosphatase 2A (PP1 and PP2A), affecting the phosphorylation or dephosphorylation of proteins and consequently a large number of cellular processes, such as metabolism, division, cytoskeletal integrity, membrane transport, gene translation (Landsberg, 2002). Because of this, they demonstrate high cytotoxic activity and ability to promote tumors, and their effects are dose-related. OA has been used to analyze the mechanisms by which the conjugated linoleic acids can act as antitumor agents in breast cancer cells. Other OA medical uses against Alzheimer's, AIDS and diabetes are also studied currently.

The availability of phycotoxin quantities for investigational purposes can be a significant limitation. For example, a temporary lack of toxin reference standards could impose severe draw-backs in safety monitoring routine controls of seafood contaminated with phycotoxins. Moreover, some of these toxins available at small amounts are quite expensive and their chemical synthesis is complex and costly. For example, currently OA and DTX1 calibrants are mainly produced from *Prorocentrum* cultures (Burton *et al.*, 2005; Crain *et al.*, 2015; Watanabe *et al.* 2016). However, mass cultures of some dinoflagellate species for the production of phycotoxins' calibrants are difficult to handle due to their slow growth and increased sensitivity, e.g. to turbulence. Because of this, mass culturing of dinoflagellates is a highly demanding and problematic task. Experimental works for mass culturing of species of *Prorocentrum* have been rather scarce so far (Murakami *et al.*, 1982; Hu *et al.*, 1992; Morton and Bomber, 1994). The aim of this study is to present the design and development of an upscale system for the production of biomass and the toxins OA and DTX1 by the marine dinoflagellate *Prorocentrum lima*.

Materials and Methods

Cultures of *P. lima*

A *P. lima* strain (CCAP 1136/11) was grown in 100 mL monospecific non-axenic batch cultures in Erlenmeyer flasks in a cool room (20 °C temperature). All growth media were prepared with natural seawater of salinity 38 ppt, collected from oligotrophic waters, filtered with 0.2 µm pore filters and autoclaved. The *f/2* growth medium (Guillard, 1975) was modified according to Anderson *et al.* (1996) and with deficient phosphorus (see below), and it was added to the autoclaved seawater.

Biomass Upscale System

After acclimation of the cells for a few generations, the upscale of *P. lima* culture volumes began and they were brought up gradually from 100 mL to 30 L in a polypropylene bag (of 35 L capacity) as semi-continuous cultures, i.e. the total volume of an initial culture (e.g. 100

mL) was inoculated into a two times higher volume of fresh culture medium (200 mL), thus making a three times higher final culture volume (300 mL) every two weeks approximately. We used 10 polypropylene bags making a total of 300 L of culture volume. The biomass upscale system was developed in a cool room. The seawater was now filtered with 10 µm, 2 µm and 0.2 µm cartridges successively and sterilized with UV radiation. Modified *f/2* medium was added to the cultures with deficient phosphorus, i.e. the initial concentrations of nitrogen and phosphorus in the cultures were approximately 1450 µM N-NO₃⁻ and 18.1 µM P-PO₄³⁻ (N:P ratio 80:1) (see in Varkitzi *et al.* 2010 for details). Culture conditions were a 16:8 light : dark cycle, a light intensity of 35 µmol photons/m² s⁻¹, provided by cool white fluorescent tubes, and 20 °C temperature. Adequate aeration was supplied constantly to the polypropylene bags, with air pumps sending atmospheric air filtered through Puradisk filters (25 mm, 0.2 µm) at a 100 L/h rate. The culture bags were shaken manually every day. The cultures were incubated for approximately 3.5 weeks.

Culture Sampling and Growth Parameters

Each polypropylene bag was vigorously shaken before sampling, in order to homogenize the cultures. Samples of 60-120 mL of culture suspension were removed from each bag on regular intervals at the same time of the day. A sub-sample of the culture suspension (approximately 10 mL) was fixed with Lugol's solution for the determination of cell densities (cells/mL) and the calculation of biomass production. Cell densities were determined microscopically with a Sedgewick Rafter 50S chamber under a NIKON DIAPHOT microscope (150X magnification) (Andersen and Thronsen, 2004). Growth rates were calculated according to the equation of Guillard (1973).

Analysis of Toxins

Sub-samples of the culture suspension (50 to 100 mL) were gently filtered (at 7-8 kPa pressure) on precombusted GF/F filters. The okadaic acid (OA) and dinophysistoxin 1 (DTX1) were analysed on the cells retained on the filters. OA and DTX1 analysis was performed with HPLC-FLD according to the method of Zhou *et al.* (1999) with modifications in the pretreatment procedure of the samples. These modifications involved: extraction with 80% methanol, liquid-liquid extraction of the methanolic extract with ammonium sulfate solution and dichloromethane, derivatization of the dry residue of the dichloromethane extract with 3-bromo-7-methyl-1,4-benzoxazin-2-one (BrMB) in alkaline environment and solid phase extraction of the derivative with 200 mg silica. The fraction with OA and DTX1 was dried under a nitrogen stream, re-dissolved in the mobile phase solvent mixture and then injected in the HPLC. The HPLC determination was performed on a Shimadzu instrument equipped with a LC-10AD ternary pump and a RF-10Ax1 fluorescence detector. The LC

column was Spherisorb ODS2, 25 cm X 4.6 mm, 5 µm and maintained at 28 °C. Isocratic conditions were used, the mobile phase was acetonitrile/water (65:35) and the flow rate 1.0 mL/min. The fluorescence detector was set to 345 nm excitation and 440 nm emission. The OA and DTX1 concentrations were calculated using two calibration curves respectively, based on injections of an OA standard solution by Calbiochem and a DTX1 standard solution by Wako. Both standard solutions were derivatized under the same conditions described here.

Statistics

Statistical analyses were computed with the STATGRAPHICS software. Pearson correlation analysis and regression analysis were performed.

Results and Discussion

The biomass upscale system for the marine toxic Dinoflagellate *P. lima* was designed and developed with success and it is presented in Fig. 1. The cultures did not collapse and the cells were dividing. Under the microscope, the cells of *P. lima* looked vivid and robust without any sign of stress. The upscale of the culture volumes reached a total of 300 L in the polypropylene bags. The total capacity of our culturing system could reach 500 L. To our knowledge, there are previous records in the literature of a biomass upscale system with 50 and 30L tanks for *P. lima* (Hu et al., 1992, and Murakami et al., 1982, respectively), and with 36L tank for *Prorocentrum hoffmannianum* (Morton and Bomber, 1994). However, the culturing capacity of 500L

that was achieved in the present work, is one of the highest mentioned so far for such a dinoflagellate upscale system.

As growth proceeded and despite the constant air supply, *P. lima* formed clumps in the bags and the Erlenmeyer flasks, which were observed under the microscope. It is a common practice for *P. lima* to form clumps on artificial or natural surfaces, eg on macroalgae thalli or sand grains (Heredia-Tapia et al. 2002; Licea et al. 2004; Nascimento et al. 2005; Varkitzi et al. 2010). As the biomass became denser, clumps also were getting denser and cell enumeration problematic. Gentle sonication of the samples with a probe (at 30% amplitude, 1.5 sec on/off pulses for 2 min) dissolved the clumps without any alteration imposed on the cells, e.g. cell wall disruption. The large scale cultures of *P. lima* were grown under phosphorus-deficient cultures because of our previous findings showing higher OA production by *P. lima* in phosphorus-deficient cultures (Varkitzi et al., 2010). Therefore, the initial concentrations of nitrogen and phosphorus in the cultures were approximately 1450 µM N-NO₃⁻ and 18.1 µM P-PO₄³⁻ (N:P ratio 80:1), as in Varkitzi et al. (2010).

The evolution of *P. lima* cell growth in large scale cultures is presented in Fig. 2. *P. lima* biomass (expressed as cell numbers per mL of culture suspension) reached high levels (max 20690 cells/mL on average). Doubling time of *P. lima* biomass was 3.7 days. Cell growth rates ranged at low levels, from 0.02 to 0.27 divisions per day (on average 0.17 ± 0.12 SD divisions per day), and continued to increase until the end of incubation, even though at low rates (day 24).



Fig. 1: The biomass upscale system for the toxic marine dinoflagellate *Prorocentrum lima* in polypropylene bags in a cool room.

P. lima is a well known slowly growing species and the levels of biomass and growth rates we found, are similar to those mentioned in the literature for smaller scale cultures (Morlaix and Lassus, 1992; Pan et al., 1999; Bravo et al., 2001; Heredia-Tapia et al., 2002; Nascimento et al. 2005). Pearson correlation coefficient between *P. lima* cell numbers and incubation time was 0.96 indicating a strong positive relation between these two variables ($p < 0.05$). Regression analysis gave the following equation, which better describes the relation of these two variables at a significant level ($p < 0.05$) with high $R^2 = 0.912$,

$$y = 635.99 + 1338.4x - 26.016x^2,$$

where x =incubation days and y =cell numbers (see Fig. 2 for regression curve).

The concentrations of the phycotoxins okadaic acid (OA), dinophysistoxin 1 (DTX1) and the total DSP toxin per culture volume during the incubation period are presented in Fig. 3. The toxins concentrations increased after day 12 of incubation and until day 21, when maximum values were reached. Maximum toxin production was 63.66 ng/mL for OA and 8.07 ng/mL for DTX1. The range of the intracellular content for the toxins OA, DTX1 and total toxin are presented Table 1. Intracellular OA reached 14.72 pg/cell and DTX1 1.87 pg/cell. The time evolution of the intracellular toxins in Fig. 4 demonstrates a very similar pattern to that of the toxins concentrations per culture volume.

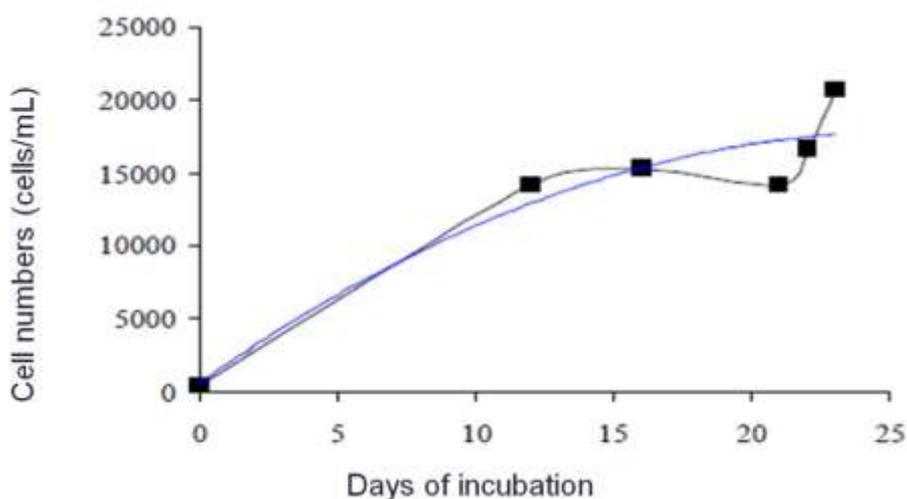


Fig. 2: Biomass production (expressed as cells/mL) by the toxic marine dinoflagellate *Prorocentrum lima* growing in the biomass upscale system.

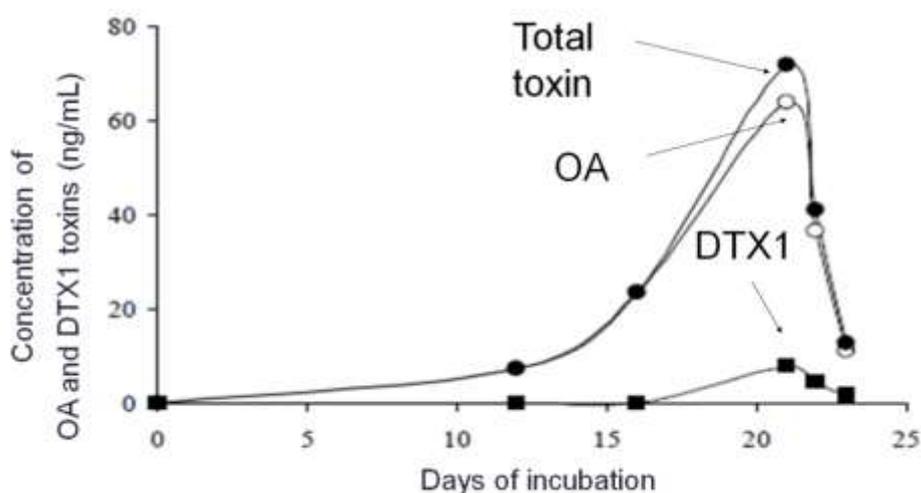
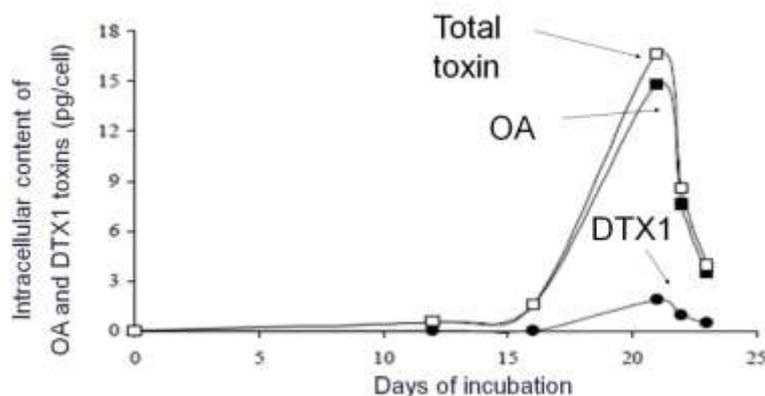


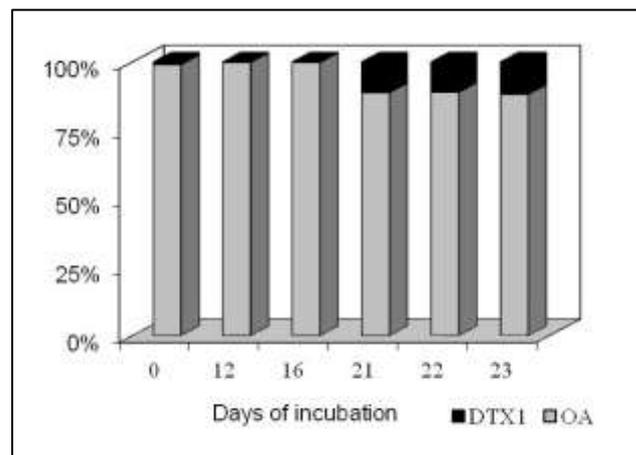
Fig. 3: The concentrations of the phycotoxins okadaic acid (OA), dinophysistoxin 1 (DTX1) and the total DSP toxin produced per culture volume (ng/mL) during the incubation period of *Prorocentrum lima* cultures in the biomass upscale system.

Table 1: Range of the intracellular content of the phycotoxins okadaic acid (OA), dinophysistoxin 1 (DTX1) and the total DSP toxin (pg/cell) during the incubation period of *Prorocentrum lima* cultures in the biomass upscale system.

	Okadaic acid (pg/cell)	Dinophysistoxin 1 (pg/cell)	Total DSP toxin (pg/cell)
Range of values	0.01-14.72	0.0001-1.87	0.01-16.59
Average values±SD	4.63±5.65	0.55±0.75	5.18±6.40

**Fig. 4:** Intracellular content of the phycotoxins okadaic acid (OA), dinophysistoxin 1 (DTX1) and the total DSP toxin (pg/cell) during the incubation period of *Prorocentrum lima* cultures in the biomass upscale system.

OA and DTX1 are known to be the main toxic compounds among others that *P. lima* synthesizes. The OA production that we measured in our biomass upscale system, was comparable to previous findings (Bravo *et al.*, 2001) (OA 0.2 - 12.9 pg/cell and DTX1 0-12.40 pg/cell) and (Nascimento *et al.*, 2005) (OA 0.42 - 17.13 pg/cell and DTX1 0.41-11.29 pg/cell) for smaller scale cultures. According to our findings, the potential toxin production in this culturing system could reach 31.83 mg OA and 4.035 mg DTX1 in 500 L of *P. lima* culture. OA comprised the largest quota of the total DSP toxin for the toxins concentrations per culture volume and for the intracellular toxins throughout the incubation period (Table 1 and Fig. 5). On average OA comprised 94.1 % and DTX1 5.9% of the total DSP toxin per culture volume for the whole incubation period. Average toxin quota inside *P. lima* cells was 88.7% OA and 11.3% DTX1. The proportion of OA to DTX1 in *P. lima* cells was nearly 10:1 and this proves to be quite common for this marine dinoflagellate according to previous findings (Marr *et al.*, 1992; McLachlan *et al.*, 1994; Pillet *et al.*, 1995). It has to be mentioned though that this intracellular toxin proportion varies widely and differs significantly among *P. lima* strains of different geographical origin (Marr *et al.*, 1992; Tomas and Baden, 1993; Morton and Tindall, 1995; Bravo *et al.*, 2001; Heredia-Tapia *et al.*, 2002; Foden *et al.*, 2005; Nascimento *et al.*, 2005).

**Fig. 5:** Intracellular toxin quota of the phycotoxins okadaic acid (OA) and dinophysistoxin 1 (DTX1) (%) during the incubation period of *Prorocentrum lima* cultures in the biomass upscale system.

In conclusion, the biomass upscale system for the toxic marine dinoflagellate *Prorocentrum lima* reached 300 L of culture and proved to be sufficient for high biomass and DSP toxins production with a volume capacity of 500 L. Therefore the potential toxin production in this culturing system could reach 31.83 mg OA and 4.035 mg DTX1. Follow-up experiments could be conducted for the isolation, purification and certification of the DSP toxins and their analogues produced in this upscale system.

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