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PRODUCTION OF OKADAIC ACID IN PROROCENTRUM LIMA THROUGH MIXOTROPHIC CULTURES WITH MACROALGAL POLYSACCHARIDES

PRODUCCIÓN DE ÁCIDO OKADAICO EN PROROCENTRUM LIMA MEDIANTE CULTIVOS MIXOTRÓFICOS CON POLISACÁRIDOS MACROALGALES

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Production of Okadaic Acid in Prorocentrum Lima Through Mixotrophic Cultures with Macroalgal Polysaccharides

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ABSTRACT

Prorocentrum lima is highly investigated in the aquaculture of bivalve mollusks due to the production of toxins. Currently, various studies with traditional autotrophic means are used to increase the production of okadaic acid (OA) in *P. lima* culture, although the synthesis of its toxins occurs in small quantities. The innovation of mixotrophic cultures with macroalgal polysaccharides allowed us to know that the *P. lima* strains used were capable of assimilating organic carbon. Strain D008-1 grew better in the medium with *Agarophyton chilensis* polysaccharides (0.07 ± 0.06 day^{-1}), however, strain D008-5 that grew in the medium with *Codium fragile* polysaccharides synthesized the greatest amount of OA (13.2 pg OA cell^{-1}) on day 7, achieving more OA in a shorter time than the L1-Si autotrophic medium. This production could be due to the presence of sulfated and glucose groups in the polysaccharides of *C. fragile*. In both autotrophic and mixotrophic cultures, a high production of OA was obtained compared to a reduced amount of Dinophysistoxin-1 (DTX-1). This study consisted of implementing a non-traditional means through an organic carbon source to increase biomass growth and toxin synthesis of *P. lima*.

Keywords: culture, benthic dinoflagellate, diarrheal toxin, macroalgal polysaccharides, mixotrophy

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Producción de Ácido Okadaico en Prorocentrum Lima Mediante Cultivos Mixotróficos con Polisacáridos Macroalgales

RESUMEN

Prorocentrum lima es muy investigada en la acuicultura de moluscos bivalvos por la producción de toxinas. Actualmente, diversos estudios con los tradicionales medios autotróficos son usados para incrementar la producción de ácido okadaico (AO) en cultivo de *P. lima*, aunque la síntesis de sus toxinas ocurre en pequeñas cantidades. La innovación de cultivos mixotróficos con polisacáridos macroalgales permitió conocer que las cepas de *P. lima* utilizadas fueron capaces de asimilar carbono orgánico. La cepa D008-1 creció mejor en el medio con polisacáridos de *Agarophyton chilensis* (0,07±0,06 día^{-1}), sin embargo, la cepa D008-5 que creció en el medio con polisacáridos de *Codium fragile* sintetizó la mayor cantidad de AO (13,2 pg AO célula-^{1}) al día 7, logrando en menor tiempo más AO que el medio autotrófico L1-Si. Esta producción pudo deberse a la presencia de los grupos sulfatados y de glucosa en los polisacáridos de *C. fragile*. Tanto en cultivos autotróficos como en mixotróficos se obtuvo una alta producción de AO en comparación con una reducida cantidad de Dinophysistoxina-1 (DTX-1). Este estudio consistió en implementar un medio no tradicional a través de una fuente de carbono orgánico para incrementar el crecimiento en biomasa y la síntesis de toxinas de *P. lima*.

***Palabras clave*:** cultivo, dinoflagelado bentónico, toxina diarreica, polisacáridos macroalgales, mixotrofía

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INTRODUCTION

In recent decades, some species of phototrophic dinoflagellates (e.g. *Takayama helix, Karenia brevis*, *Alexandrium catenella, A. minutum, Heterocapsa rotundata, P. minimum*, *P. donghaiense, P. micans*, etc.), have been identified as organisms that have the ability to obtain their energy through mixotrophy (Jeong et al., 2005; 2010; 2015; 2016; Lee et al., 2016). These dinoflagellates that use mixotrophic feeding represent a small proportion of the total populations of phototrophic dinoflagellates (Gómez, 2012; Lee et al., 2014; Jeong et al., 2016). These species commonly inhabit shallow waters, attached to different biotic and abiotic substrates (Lee et al., 2020), allowing the development of a complex epiphytic community, where they can interact with other microorganisms such as bacteria, fungi and other types of microalgae (Florez et al., 2017; Zhang et al., 2009; Tiffany et al., 2011). The benthic dinoflagellate *Prorocentrum lima* is frequently found attached to these substrates and due to this strong adhesion, its dispersion in adjacent environments is very difficult, so its nutrition constantly depends on these substrates. Furthermore, as these benthic species tend to be more flattened with respect to planktonic dinoflagellates, their surface/volume ratio is favored, facilitating the absorption of nutrients in oligotrophic conditions (Fraga, 2014) and being in direct contact with the coastal seabed and not depending on the water column to obtain most of the nutrients, it can be considered a species with the ability to develop under different nutritional conditions.

P. lima, has been the most researched microalgae with the purpose of being cultivated, since in addition to being one of the first species confirmed as causing Diarrheal Poisoning Syndrome (DPS) in mollusks (Murakami et al., 1982), it has been linked to various episodes of this poisoning in mollusks grown in various farms (Lawrence et al., 2000; Levasseur et al., 2003) with case reports annually, which shows its health risk worldwide (Nascimento et al., 2016).

Parsons & Preskitt, 2007; Tester et al., 2014, mentioned that *P. lima* is frequently found on various species of macroalgae such as *Codium fragile*, *Ulva* spp., *Agarophyton chilensis* among others, in addition to rocky substrates and sand, being able to take advantage of the nutrients coming from these habitats. This ability to be able to use both autotrophic and possibly mixotrophic feeding; would influence its physiology, growth and the increase in its secondary metabolites, such as okadaic acid (OA) and its analogues (Mitra & Flynn, 2010; Zhang et al., 2013; Jeon et al., 2015), compounds

currently in high demand as standards for chromatographic analysis (Suzuki et al., 2014), which makes it a candidate to be cultivated under nutritional conditions typical of benthic organisms. These conditions could offer certain advantages, such as growing with limited light, depleted nutrients or high concentrations of organic matter (Salerno & Stoecker, 2009; Riebesell et al., 2017). This species could use organic matter during periods of darkness, while inorganic matter would be in the presence of light and it could even occur that both sources are assimilated concomitantly (Jeong et al., 2005).

Currently, there are several studies in which crops have been carried out with autotrophic media, to increase the production of OA in *P. lima*, varying proportions of macronutrients such as nitrogen and phosphorus (McLachlan et al., 1994; Vanucci et al., 2010; Varkitzi et al., 2010; Varkitzi et al., 2017), thermal variations (McLachlan et al., 1994; Koike et al., 1998; Aquino-Cruz et al., 2018), salinities (Wang et al., 2015), irradiation and photoperiod (Vanucci et al., 2010; Wang et al., 2015; Aquino-Cruz et al., 2018; David et al., 2018), providing valuable information for understanding the cultivation of this dinoflagellate.

There is little background on the effects of different organic compounds and extracellular products exuded by macroalgae that can promote the growth and development of microalgal species (Carlson et al., 1984; Bomber et al., 1989; Rains & Parsons, 2015). Wang et al., (2012), reported that the organic matter from the *Ulva* macroalga proliferates in decomposition, produced the development, and increase of species that cause Harmful Algal Blooms (*Heterosigma akashiwo, Alexandrium tamarense, P. donghaiense* and *Skeletonema costatum*). Also, Santelices & Varela, (1993), mentioned that the exudates of total polysaccharides of *A. chilensis* in a medium stimulate the settlement and recruitment of species such as *Ulva lactuca*, which in *P. lima* could similarly favor its development. Likewise, Shalaby & Amin, (2019), mentioned the possible nutritional value of the sulfated polysaccharide, ulvano, obtained from different extracts of *U. lactuca*, which could be used by *P. lima* to increase its cell density. This work aims to evaluate the growth and production of OA and derivatives of the benthic dinoflagellate *P. lima*, in mixotrophic culture media based on the polysaccharides of the macroalgae *C. fragile, A. chilensis* and *Ulva* spp., in comparison with the traditional autotrophic medium, L1 without silicate (L1-Si).

MATERIAL AND METHODS

Collection of macroalgae and extraction of macroalgal polysaccharides

A collection of macroalgae (*A. chilensis*, *Ulva* spp. and *C. fragile*) was carried out in La Herradura Bay, Coquimbo, Chile (29°58'44"S and 71°21'8"W). The samples were immediately washed with distilled water to remove microepibionts, remains of sand and salts. Subsequently, the algae were dried in an LS Series Freeze Dryer and pulverized with a mortar. For the extraction of macroalgal polysaccharides (PsMA), 20 g of dry powder of each species of macroalgae were taken and depigmented using 200 mL of 96% ethanol (Sigma-Aldrich), shaking them in a Quimis Q261A21 shaking plate. The extract obtained was centrifuged for 5 minutes at 20000× g and 8ºC, using a Centurion K2015R refrigerated centrifuge (Centurion Scientific Limited). The pellet obtained was mixed with 200 mL of distilled water and stirred for 30 minutes on a heating plate with stirring at 90ºC (Quimis, SH-3). The polysaccharides (Ps) obtained in the extract were precipitated by adding 200 mL of 96% ethanol (v/v) for 24 hours (Sun et al., 2014). Subsequently, the extract was centrifuged at 20000× g for 5 minutes at 8°C. This pellet obtained was mixed with 100 mL of 4 M NaCl (Sigma-Aldrich) with stirring and a temperature of 90ºC until completely dissolved. Once cooled, 200 mL of 96% ethanol in a (v/v) extract/alcohol ratio were added for 24 h. They were then centrifuged at 20000× g for 5 minutes to 8°C. Subsequently, the pellet containing the total Ps and the salts from the 4 M NaCl solution were placed on a dialysis membrane (Sigma-Aldrich) and in a 0.5 M NaCl solution for 24 h. Finally, the solution was centrifuged at 20000× g for 5 minutes at 8°C, the resulting pellets were dried in an oven at 40°C for 24 h and pulverized with a mortar.

Determination of the concentration of treatments with macroalgal polysaccharide media (15, 50 and 100 mg L^{-1}) in *P. lima* strain D008-5

Triplicate cultures of strain D008-5 were performed with approximate inocula of 4,000 cells mL^{-1} in 250 mL Erlenmeyer flasks with 100 mL^{-1} of the L1-Si media (Guillard & Hargraves, 1993) plus *A. chilensis* polysaccharides (PsACH) at 15 mg. L^{-1}, 50 mg L^{-1} and 100 mg L^{-1}; polysaccharides from *Ulva* spp. (PsULV) at 15 mg L^{-1}, 50 mg L^{-1} and 100 mg L^{-1}; *C. fragile* polysaccharides (PsCFR) at 15 mg L^{-1}, 50 mg L^{-1} and 100 mg L^{-1} and in L1-Si without PsMA as a control medium, for 18 days. All cultures were maintained at a temperature of 20 °C, irradiation of 80 µmol of photons $m^{-2}s^{-1}$ and photoperiod of

8 h: 16 h (light/dark). Every three days, 1 mL samples were taken from each culture to record the experimental cell densities achieved. Growth curves and standardization of growth rates for each treatment were estimated by calculating cell densities every three days using the method described by LeGeresley & McDermott, 2010. Once these values are obtained the daily growth rates (day^{-1}) were estimated (Levasseur et al., 1993), using the following formula:

$$\mu = \frac{\ln(N2/N1)}{(t2-t1)} \quad (1),$$

where N2 is the final concentration (cells mL^{-1}), N1 is the initial concentration (cells mL^{-1}), t2 is the final time (day) and t1 is the initial time (day).

Additionally, with the values of the experimental cell densities, the maximum growth rates and maximum cell concentrations were estimated, using a mathematical model of exponential growth with saturation, from the initial observation (day 0). The observed experimental data were fitted according to the equation:

$$G=Gm-A*\exp(-\mu m*(t-\lambda)) \quad (2),$$

where G is the cell concentration (cells mL^{-1}), Gm is the maximum cell concentration (cells mL^{-1}), A is the inoculum (cells mL^{-1}), μm is the maximum specific growth rate (day^{-1}), t is the cultivation period (day) and λ is the lag phase (day).

Numerical modeling of the growth data was performed using the nonlinear least squares method of the "Solver" macro of the Microsoft Excel spreadsheet and the error associated with the parameter estimates (as confidence intervals) was calculated and evaluated using Student's t test. With the theoretical data obtained in the program and with the experimental data, the growth curves were generated and compared. The experimental results (observed) and the theoretical results (expected) of the mathematical model were statistically analyzed with one-way analysis of variance (ANOVA) for each strain in each culture, performing Fisher's post-hoc test using the statistical program Statistics (StatSoft, Inc. 2005). The variability of the maximum cell densities, the theoretical maximum growth rates obtained in the mathematical model, and the variability of the experimental cell densities and growth rates were then evaluated.

Once this concentration of PsMA was selected, strain D008-1 was also cultured in each of the media with the three PsMA at the concentration selected as the best treatment in strain D008-5 and under the same culture conditions for 15 days. Finally, both strains were acclimated to these media with inoculum of approximately 10,000 cells mL^{-1} for the growth test of both strains in macroalgal polysaccharide media (50 mg L^{-1}).

Growth of *P. lima* strains D008-1 and D008-5 in macroalgal polysaccharide media (50 mg L^{-1})

For the cultivation of *P. lima* strains D008-1 and D008-5 in mixotrophic media with macroalgal polysaccharides (PsMA), 25 mL of L1-Si media, plus PsACH at 50 mg L^{-1}, PsULV 50 mg L^{-1} and PsCFR 50 mg L^{-1}, in addition to L1-Si without PsMA (control), were transferred into glass test tubes with screw caps. In these media, both strains acclimated to the media with PsMA were inoculated in triplicate, with an initial density of between 9,000 and 12,000 cells mL^{-1}. These strains grew for 18 days under the same culture conditions indicated in the previous section. From these cultures in the test tubes, samples of 1 mL of each strain were taken in triplicate in the different media, on days 3, 5, 7, 12, 15 and 18, and the cell densities of each sample were obtained. The growth curves for each treatment and strain were estimated with the mathematical growth model described above.

Production of Okadaic Acid (OA) and Dinophysistoxin-1 (DTX-1) of *P. lima* strains D008-1 and D008-5 in culture media of 50 mg L^{-1} of macroalgal polysaccharides (PsMA)

The 24 mL samples of each of the cultures in the test tubes were concentrated by centrifuging at 20000× g for 10 minutes, using a refrigerated centrifuge (4ºC) (Centurion K2015R, Centurion Scientific Ltd., Stoughton, West Sussex, UK). The supernatants were discarded, and each resulting cell pellet was resuspended in 2 mL of 100% methanol, sonicated for two minutes in pulse mode (50% duty cycle, 375 watts) in a Branson 150D ultrasonic homogenizer (Branson Ultrasonic Corp), while cooling in an ice bath. Each extract was separated by centrifugation at 18.973× g for 10 minutes. The supernatants were filtered through syringes with a PTFE syringe filter (0.22 µm) (Jet Bio-Filtration Co., Ltd). Subsequently, they were placed in amber chromatographic vials and stored at -20ºC until analysis. Before analysis, each sample was hydrolyzed to esterify the toxins that were not free and thus obtain the total concentrations of OA and DTX's, following the protocol of Mountfort et al., (2001) using 0.5 mL of the sample, adding 62.5 µL of $NaOH_4$ (2.5 M) and mixing with vortex for 30 seconds.

Subsequently, it was heated to 76°C for 40 minutes, cooled to room temperature and neutralized with 62.5 mL of HCl (EU-RL-MB, (2015). The identification and quantification of okadaic acid (OA), dinophysistoxin-1 (DTX1) and dinophysistoxin-2 (DTX2) for these strains was carried out by Ultra High Pressure Liquid Chromatography (UHPLC) and detection by high resolution mass spectrometry (HRMS). The chromatographic analysis was developed following the methodology described by (Regueiro et al., 2011) with small modifications. The chromatographic system corresponded to a Dionex Ultimate 3000 chromatograph (UHPLC) (Thermo Fisher Scientific, Sunnyvale, CA, USA). Chromatographic separation was performed using a Gemini-NX C18 reversed-phase column (100 x 2.0 mm, 3µm) with an Ultra Guard C18 precolumn (Phenomenex; Torrance, California, USA) maintained at 40°C. The mobile phases consisted of 6.7 mM NH_4OH in MilliQ water (phase A); and 100 % ACN with 6.7 mM NH_4OH (phase B). The flow of the mobile phases was maintained at 0.35 mL/min. The gradient used was as follows: 15% phase B maintained for 1 minute, followed by a linear increase over 2.85 minutes until reaching 80% phase B, an increase to 85% Phase B in 0.15 minutes, 90% B for 0.75 minutes and 100% B for 3.25 minutes. Finally, the gradient returned to initial conditions in 2 minutes. Detection of lipophilic toxins was carried out using a Q Exactive Focus mass spectrometer with HESI-II electrospray interface (Thermo Fisher Scientific, Sunnyvale, CA, USA). The HESI was operated in negative ionization mode with a spray voltage of 3 kV. The temperature of the transfer tube and HESI vaporizer was 200 and 350°C, respectively. At the source, nitrogen (> 99.98%) was used as sheath gas and auxiliary gas with pressures of 20 and 4 arbitrary units, respectively. Data were acquired in SIM (Selected Ion Monitoring) mode and data acquisition in ddMS2 (data dependent) mode. In SIM mode, the scanning range was from 100 – 1000 m/z with a resolution of 70,000, an automatic gain (AGC) of 5 x 104 and a maximum injection (IT) of 3,000 ms. For dds2 the mass resolution was 70,000, the AGC was 5 x 104 and a maximum injection (IT) of 3,000 ms. In both cases the isolation window was 2 m/z. For each compound of interest, the exact mass of the precursor ion, retention time, and collision energy were included. Toxins were identified based on retention time and mass spectra in comparison with calibration curves and mass spectra of external standards. The toxin concentration in the extracts of each strain was quantified by comparing the area and peaks obtained in the chromatograms with those of the certified reference materials obtained NCR, Canada. Finally, the toxin content was calculated per

cell and then at the level of each 24 mL culture. The experimental results were statistically analyzed with one-way analysis of variance (ANOVA) for the selected days in each crop, performing Tukey's post-hoc test, from the Statistica statistical program (StatSoft, Inc. 2005) to evaluate the variability of cell densities, growth rates and production of OA and DTX1 between treatments.

RESULTS AND DISCUSSION

Determination of the concentration of treatments with macroalgal polysaccharide media in *P. lima* strain D008-5

The cultures of *P. lima* strain D008-5 in the L1-Si media, plus the three concentrations (15, 50 and 100 mg L^{-1}) of polysaccharides (Ps) from *A. chilensis* (PsACH) presented similar growth the first 12 days of cultivation (Fig. 1). Then they began to differentiate, reaching the highest experimental cell density of 29,913 ± 1,255 cells mL^{-1} in the treatment of 50 mg L-1 of PsACH after 18 days. Their experimental growth rates were between 0.1051 – 0.1152 day^{-1}, obtaining close values when comparing the experimental results with the theoretical ones in each treatment.

This D008-5 strain in cultures with L1-Si media, plus the three concentrations (15, 50 y 100 mg L^{-1}) of Ps from *Ulva* spp. (PsULV), also presented similar growth the first 12 days of culture. After that time, no differentiation was observed in their growth, reaching a maximum experimental cell density of 27,043 ± 4,553 cells mL^{-1} in the treatment of 50 mg L^{-1} of PsULV at 18 days of culture (Fig. 1). These cultures in PsULV obtained experimental growth rates between 0.1007 – 0.1061 day^{-1}. Close values were also obtained when comparing the experimental results with the theoretical results in each treatment.

Finally, in the cultures with the L1-Si media, plus the three concentrations (15, 50 and 100 mg L^{-1}) of Ps from *C. fragile* (PsCFR), likewise, strain D008-5 presented similar growth in the first 12 days of culture (Fig. 1). Subsequently, no differentiation was observed in their growth, with a maximum experimental cell density of 27,253 ± 1,848 cells mL^{-1} in the treatment of 50 mg L^{-1} of PsCFR at the end of the culture. The cultures in PsCFR obtained experimental growth rates between 0.0984 – 0.1062 day^{-1}. Similarly, close values were obtained when comparing the experimental results with the theoretical results in each treatment.

The lowest experimental density among all cultures was obtained in L1-Si at the end of the experiment (23,309 ± 796 cells mL^{-1}) with experimental growth rates of 0.0975 ± 0.0270 day^{-1} (Fig. 1).

P. lima strain D008-5 was grown with mixotrophic media (L1-Si + 15, 50 and 100 mg L^{-1} of PsMA), presented experimental average growth rates, like and higher (0.09 – 0.11 day^{-1}) than the L1-Si control (0.09 day^{-1}). These growth rates were lower than those reported by Morton & Tindall, (1995) (0.20 day^{-1}) and similar to Nascimento et al., (2005) (0.11 day^{-1}), which differed in the base culture medium.

The maximum experimental cell density of *P. lima* was recorded in the treatment with 50 mg L^{-1} of all PsMA (*A. chilensis, C. fragile* and *Ulva* spp.), and now no studies have been found that relate the cultivation of this species and the addition of PsMA to increase its biomass.

The crops to which PsMA were added showed better growth (> 13%) compared to L1-Si, which indicates the ability of *P. lima* to assimilate an organic carbon source, pointing out that *P. lima* is not an exclusively autotrophic species but rather mixotrophic. Glibert & Legrand, (2006), mention that some dinoflagellates are capable of producing extracellular enzymes that hydrolyze macromolecules in the aquatic environment, to be absorbed by these cells (Cembella et al., 1985), or through an enzymatic action developed on its cell surface (Stoecker & Gustafson, 2003). In this context, little is known about the enzymatic profiles in benthic dinoflagellates, but transcriptomic studies have shown that *Gambierdiscus caribaeus* has α and β galactosidases that allow the growth of this species through the use of galactose, in laboratory cultures (Price et al., 2016).

Matsuhiro & Urzúa, (1990), reported that *A. chilensis* is made up of galactose molecules with similar percentages for 3,6-anhydrous galactose (33.5%) and sulfated galactans (2.3%), in addition to the presence of galactose (32.2%), 6-0 Methylgalactose (6.5%) and pyruvic acid (0.16 %). These polysaccharides would provide a source of organic carbon that would be assimilated by *P. lima* cells through their enzymatic actions. The enzymatic reactions produced by agarases and carrageenases would be very relevant during the mixotrophic metabolic process from polysaccharides, since they would trigger the biosynthesis of biologically active components that would form their cellular structures, promoting the growth of this dinoflagellate.

The highest theoretical cell density of *P. lima* obtained with the treatment of 50 mg L^{-1} of the Ps of *A. chilensis, C. fragile* and *Ulva* spp., presented significant differences ($p < 0.05$) with the concentrations

of 15 mg L^{-1}, 100 mg L^{-1} and the autotrophic medium L1-Si, results that allowed selecting the treatment of 50 mg L^{-1} for the media with PsMA in mixotrophic cultures.

Growth of *P. lima* strains D008-1 and D008-5 in macroalgal polysaccharide media (50 mg L^{-1})

On day 18, strain D008-1 presented the highest experimental cell densities in PsCFR (32,800 ± 1,924 cells mL^{-1}) and in PsULV (32,320 ± 520 cells mL^{-1}). The lowest experimental cell density (28,850 ± 926 cells mL^{-1}) was obtained in L1-Si ($p < 0.05$). The highest experimental growth rate was obtained in PsACH (0.0726 ± 0.0615 day^{-1}) and the lowest in L1-Si (0.0560 ± 0.00537 day^{-1}). Close values were obtained when comparing the experimental results with the theoretical results in each treatment.

The D008-5 strain obtained the highest experimental densities in the cultures with PsACH (29,724 ± 254 cells mL^{-1}) ($p < 0.05$), followed by PsULV (27,235 ± 1,310 cells mL^{-1}), both on day 18. The lowest experimental maximum cell density (22,250 ± 601 cells mL^{-1}) was obtained in L1-Si. The maximum experimental growth rate was obtained in PsACH (0.0682 ± 0.0448 day^{-1}) and the lowest in L1-Si (0.0498 ± 0.0397 day^{-1}) Close values were also obtained when comparing the experimental results with the theoretical ones in each treatment.

The maximum cell density obtained in strain D008-1 (32,800 ± 1,924 cells mL^{-1}), was produced with the PsCFR medium, coinciding with the fact that this strain was isolated from *C. fragile*, giving a species-specific physiological condition (Bravo et al., 2001), of the host and the Ps. This relationship did not occur with strain D008-5 since it obtained lower cell densities (23,182 ± 2,781 cells mL^{-1}) in this same culture medium. This strain was isolated from the surface of the valves of *A. purpuratus*.

When macroalgal sugars are added to the inorganic culture medium, they would provide a source of organic carbon, whose absorption would be favored over that produced in photosynthesis when mixotrophic conditions exist in a culture, synthesizing compounds typical of photosynthetic and heterotrophic metabolisms (Smith & Gilmour, 2018), increasing growth rates and microalgal biomass concentration (Abreu et al., 2012). In the present study, it was observed that mixotrophic cultures with the addition of organic carbon sources from macroalgal polysaccharides favored (> 13%) the cellular increase of *P. lima* strains D008-1 and D008-5.

Production of Okadaic acid (OA) and Dinophysistoxin-1 (DTX-1) of *P. lima* strains D008-1 and D008-5 grown on macroalgal polysaccharides (PsMA)

- **OA and DTX-1 content per cell**

The D008-1 strain recorded its highest OA content per cell in the L1-Si medium (8.42 ± 2.33 pg OA cell^{-1}), while the lowest was in PsACH (6.11 ± 0.92 pg OA cell^{-1}). *P. lima* strain D008-5 recorded its highest OA content per cell with PsCFR (11.95 ± 1.98 pg OA cell^{-1}), while in L1-Si (6.12 ± 0.54 pg OA cell^{-1}) the lowest value was obtained. During the study, *P. lima* strain D008-1 presented the highest content of DTX-1 in the L1-Si medium (0.0198 ± 0.0034 pg DTX-1 cell^{-1}) and the lowest in PsACH (0.0108 ± 0.0013 pg DTX-1 cell^{-1}). *P. lima* strain D008-5 obtained the highest content of DTX-1 in the PsCFR medium (0.0298 ± 0.0016 pg DTX-1 cell^{-1}) ($p < 0.05$) and the lowest in PsACH (0.0140 ± 00024 pg DTX-1 cell^{-1}).

- **Amount of OA per culture**

Strain D008-1 obtained the highest experimental value of OA production in the culture in L1-Si (5,829.24 ng OA) on day 18, while the lowest experimental value of OA was obtained in the PsCFR culture (4,358.74 ng OA) of the same day. Statistical tests showed no differences ($p > 0.05$) between the maximum experimental amounts of OA produced, in any of the treatments throughout the study (Table 1). Cultivation in L1-Si for strain D008-1 obtained an experimental synthesis of OA, 1.28; 1.34 and 1.27 times higher than in PsULV, PsCFR and PsACH cultures, respectively; during the test. This strain failed to produce high concentrations of OA when grown with PsMA, synthesizing the greatest amount in the L1-Si autotrophic medium.

For its part, the strain D008-5 obtained the highest values in experimental OA production in mixotrophic cultures with PsMA. PsULV began to produce the highest amounts of OA from day 12, obtaining the highest experimental value (5,529.76 ng OA) on day 18. PsCFR also produced a higher value (4,769.63 ng OA) on day seven, while PsACH (4,160.36 ng OA), on day 12. This strain in the autotrophic culture L1-Si obtained 2,959.04 ng OA ($p < 0.05$), on day 15. Close values were recorded when comparing the experimental results with the theoretical results in each treatment (Table 1). The cultures in PsULV, PsCFR and PsACH for strain D008-5 obtained an experimental synthesis of OA, 1.87; 1.61 and 1.41 times higher, respectively, than in the culture in L1-Si, during the test.

These results would indicate that cultivating *P. lima* under this type of mixotrophic conditions would induce the production of high amounts of OA in a shorter cultivation time compared to traditional autotrophic media. But the results could vary in some *P. lima* strains, as occurred in D008-1, which synthesized a lower amount of OA compared to D008-5.

Although strain D008-1, when cultivated in PsMA, did not assimilate them in a similar way or obtain high concentrations like D008-5, the results for each of the cultures were significantly similar ($p > 0.05$) throughout the test.

The lower OA production of strain D008-5 obtained in PsACH would be due to the lack of glucose in the Ps of *A chilensis*, which does not occur in *C. fragile* and *Ulva* spp., (Matsuhiro & Urzúa, 1990; Kaeffer et al., 1999; Yaich et al., 2014; Kolsi et al., 2017; Wang et al., 2020), a situation that could indicate that glucose is an essential sugar within the metabolic pathways of this strain of *P. lima* to be able to synthesize a greater amount of OA in culture. Another factor would be the sulfate groups that are present in the Ps of *C. fragile* and *Ulva* spp., since they would provide a greater amount of sulfate molecules available for the formation of sulfated diesters, which could subsequently be transformed to diol-ester in a few minutes, as a precursor to OA (Quilliam & Ross, 1996; Suzuki et al., 2004). According to Quilliam et al., (1996), these sulfated diesters would be a means of storing toxins within the cell of dinoflagellates. Subsequently, Hu et al., (2017) point out a supposed hydrolysis of sulfated diesters that would be carried out in two steps mediated by specific enzymes (esterases) of sulfated diesters (such as DTX-4 and DTX-5), initially leading to the formation of diol esters and ultimately to the release of free OA. This would favor the cellular integrity of *P. lima*, providing self-protection in the main structure of the free acids of OA, through the insertion of oxygen in the extended side chain of these sulfated dieters (Bravo et al., 2001), since the sulfated esters are essentially inactive against phosphatases PP1 and PP2A.

What happened in these processes would occur in the mixotrophic cultures of *P. lima*, Ps of *C. fragile* and *Ulva* spp., as observed in the present work. Furthermore, all of this will depend on each type of *P. lima* strain, since each of them presents a certain type of variability to the different conditions in which they are grown (Bravo et al., 2001).

- **Amount of DTX-1 per culture**

The highest experimental amount of DTX-1 produced by strain D008-1 was obtained in the L1-Si culture (13.77 ± 2.67 ng DTX-1) on day 18, while the lowest amount was produced in PsACH (7.16 ± 2.17 ng DTX-1) on day 15 (Table 1). Strain D008-5 obtained the highest experimental amount of DTX-1 by culture in L1-Si (12.22 ± 1.41 ng DTX-1) on day 15, while the lowest was obtained in culture in PsACH (8.22 ± 0.91 ng DTX-1) ($p < 0.05$), on day 18 (Table 1).

Both strains (D008-1 and D008-5) in all mixotrophic treatments with PsMA obtained low DTX-1 contents, with values that fluctuated between 0.0114 and 0.0297pg DTX-1 cell^{-1}, compared to values from studies with treatments autotrophic such as those of Wang et al., (2015) in f/2, Nishimura et al., (2020) in Daigo IMK medium or Wu et al., (2020) in f/2-Si, which recorded higher contents between 16.58 – 70.73 pg DTX-1 cell-¹. The values of DTX-1 compared to those of OA can have very significant variations at the cellular level, which will depend on the different strains of *P. lima* that produce them. Luo et al., (2017) isolated eight strains of *P. lima* in Beihai, China and one in Kending, Taiwan, which when grown at f/2, only two presented DTX-1 (0.91 – 1.81 pg DTX-1 cell-¹), while this toxin was not detected in six of them, although OA was detected in all strains (0.55 – 10.26 pg OA cell-¹). This variability between the concentrations of OA and DTX-1 of strains D008-1 and D008-5 could probably be due to the influence of environmental, physical, physiological and genetic factors, as pointed out by Aquino-Cruz et al., (2018) and Niu et al., (2019).

Nascimento et al., (2005) when analyzing 20 strains isolated in the south of England from the Fleet Dorset Lagoon, found that DTX-1 values fluctuated between 0.41 – 11.29 pg cell^{-1}, while those of OA were between 0.42 and 17.13 pg cell^{-1}. Similarly, in the study by Wu et al., (2020) carried out in China, of five strains analyzed, three presented a ratio of lower amounts of total DTX-1 with respect to total OA (14.45 – 32.54 pg of OA cell-¹ and 0.69 – 4.40 pg of OA cell-¹); relationship similar to that obtained in the present study for strain D008-5 (0.0298 pg DTX-1 cell-¹ and 11.95 pg of OA cell-¹). Studies such as those by de Pan et al., (1999); Nascimento et al., (2016) and Pan et al., (2017), have shown that the highest production of these toxins (OA and DTXs) are related to the growth phases of *P. lima*, where the highest production of these polyketides occurs in the stationary phase.

However, their results differ from those obtained by Wu et al., (2020), who recorded the highest concentrations of OA and DTX-1 before the stationary stage, and also differ from the results of the present study in mixotrophic culture (polysaccharides), where the highest OA values were obtained from the exponential phase, which began on day seven of culture for strain D008-5. These quantities remained stable throughout the study with very high values in all mixotrophic media with PsCFR (11.95 pg of OA cell-¹ and 0.0298 pg DTX-1 cell-¹), compared to those obtained by Nascimento et al., (2005) that were between 0.14 – 3.20 pg of OA cell-¹ in the L1-Si autotrophic medium.

The L1-Si medium with polysaccharides from intertidal macroalgae, developed in this work, presented better results, both in growth of *P. lima* and in OA production, than autotrophic media. It could be expected that this mixotrophic medium could further raise the OA concentrations of the different strains of *P. lima* grown with autotrophic mediums and that have been mentioned in the present study. Especially the strain presented by Nishimura et al., 2020 that presented high amounts of OA and DTX-1 (13.39 and 55.27 pg cell-¹, respectively).

Figura 1

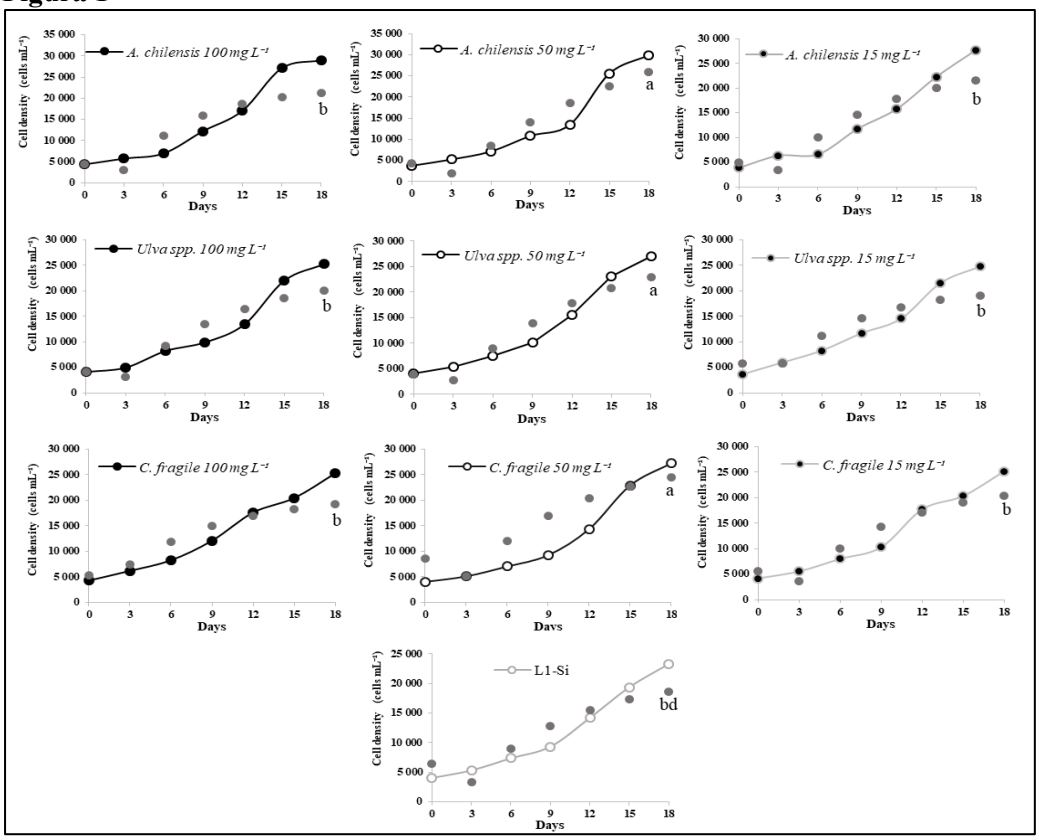

Fig. 1. Experimental (lines with markers) and theoretical (gray markers) growth of *Prorocentrum lima* strain D008-5 grown in 100 mL of the media at 15, 50 and 100 mg L^{-1} of the polysaccharides of *Agarophyton chilensis*, *Ulva* spp. and *Codium fragile*, in addition to L1-Si. Different letters below the curves indicate differences with the other theoretical treatments ($p < 0.05$) (n=3) throughout the culture.

Table 1. Maximum quantities of experimental and theoretical toxins produced by strains D008-1 and D008-5 of *Prorocentrum lima* in the 24 mL cultures of macroalgal polysaccharide media (50 mg L^{-1}) and in L1-Si. Different letters indicate differences with the other crops ($p < 0.05$) (n=3) throughout the crop.

Strain	Medium	Experimental toxin (ng culture)		Theoretical toxin (ng culture)		R^2	
		OA	DTX-1	OA	DTX-1	OA	DTX-1
D008-1	PsULV	4,568.09 ± 270.64 [a]	7.35 ± 0.22 [b]	4,348.33 ± 154.15 [a]	5.23 ± 0.10 [b]	0.9386	0.5078
	PsCFR	4,358.74 ± 1,028.57 [a]	8.03 ± 1.19 [b]	4,136.03 ± 397.56 [a]	4.11 ± 0.60 [c]	0.9906	0.4825
	PsACH	4,603.61 ± 1,585.24 [a]	7.16 ± 2.17 [b]	4,184.00 ± 106.72 [a]	4.31 ± 0.30 [bc]	0.942	0.0419
	L1-Si	5,829.24 ± 1,644.63 [a]	13.77 ± 2.67 [a]	3,821.33 ± 113.45 [ab]	7.60 ± 0.46 [a]	0.7108	0.8763
D008-5	PsULV	5,529.76 ± 1,415.40 [a]	11.69 ± 2.86 [a]	5,271.19 ± 153.02 [a]	8.62 ± 0.05 [b]	0.9675	0.7706
	PsCFR	4,769.63 ± 932.30 [a]	11.82 ± 0.70 [a]	4,565.38 ± 716.16 [a]	7.80 ± 0.45 [c]	0.8020	0.4322
	PsACH	4,160.36 ± 1,732.47 [a]	8.22 ± 0.91 [b]	3,944.33 ± 95.21 [ab]	5.31 ± 0.41 [d]	0.9166	0.9408
	L1-Si	2,959.04 ± 356.61 [ab]	12.22 ± 1.41 [a]	2,693.78 ± 98.22 [c]	9.57 ± 0.37 [a]	0.8587	0.3481

CONCLUSION

The addition of polysaccharides extracted from macroalgae to traditional *P. lima* culture media provides a source of organic carbon that stimulates the increase in its biomass, in addition to increasing the synthesis of the diarrheal toxins okadaic acid and dinophysistoxin-1. Regarding the growth of *P. lima* strains, the highest cell densities were obtained in media with *C. fragile* and *Ulva* sp. polysaccharides for D008-1, and in *A. chilensis* and *Ulva* sp. for D008-5. The lowest cell densities for both strains were obtained in the autotrophic medium L1-Si. However, the high production of these diarrheal toxins depends on the *P. lima* strain cultivated and the macroalgae species used to extract the polysaccharides. It was found that strain D008-5, when grown in media with the polysaccharides of *Ulva* sp. and *C. fragile*, obtained a high production of OA per culture, although in strain D008-1 it was higher in the Li-Si culture. Synthesis of the diarrheal toxin DTX-1 was higher in strain D008-1 in L1-Si media, with strain D008-5 showing increased production in media containing *Ulva* sp. and *C. fragile* polysaccharides, although there were no significant differences with the Li-Si medium. It is expected that mixotrophic cultures with macroalgal polysaccharides could increase OA concentrations in any strain that produces high amounts of these toxins, and even with other macroalgal polysaccharides.

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