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Effect of Dilution Rate on *Azadinium spinosum* and Azaspiracid (AZA) Production in Pilot Scale Photobioreactors for the Harvest of AZA1 and -2

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Abstract:

Azadinium spinosum, a small dinoflagellate has recently been discovered and identified as the primary producer of azaspiracid-1 (AZA) and -2. Since AZA poisoning has been reported following consumption of contaminated shellfish it is important to have these toxins available for toxicological studies, and a sustainable production of AZAs as calibrants in monitoring programs without having to rely on natural events.

In order to address this concern, continuous pilot scale cultures were carried out to evaluate the feasibility of AZA production from *A. spinosum*. Algae were cultured using two 100 L chemostats in series (R1 and R2), with agitation and pH control. Four different dilution rates were tested (0.15, 0.2, 0.25 and 0.3 day^{-1}) to evaluate chemostat bioreactors in terms of cell and toxin productivity. Algae were collected in a 300 L transparent cylindro-conical tank and harvested with a tangential flow filtration device. Subsequently, toxins were extracted from the algal retentate and separately from the permeate using solid phase adsorption procedures.

The cell concentration at steady state remained stable using different dilution rates (190,000 and 210,000 cells · mL^{-1} in R1 and R2 respectively). However, the AZA cell quota decreased as the dilution rate increased, consequently an optimum production was obtained at 0.25 day^{-1} under the studied conditions. After filtration, 50–70 % of the toxin was contained in the retentate and 30–50 % was released into the permeate. After optimization, the procedures for solid phase extraction of toxins from the retentate and permeate allowed for the recovery of 80 ± 5 % of original toxins produced. This work demonstrated the feasibility of producing AZAs from *A. spinosum* produced in a bioreactor for purification and production of certified standards.

1. Introduction

In 1995 the first azaspiracid (AZA) shellfish poisoning occurred in the Netherlands with symptoms similar to diarrhoeic shellfish poisoning (McMahon and Silke, 1996). A few years later, the toxin was identified and named azaspiracid (Satake et al., 1998) and then structurally revised (Nicolaou et al., 2004). Afterwards, a large number of analogues were identified in mussel tissues using biological assay and chemical analysis including liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), i.e. AZA2-32 (Diaz Sierra et al., 2003; James et al., 2003; McCarron et al., 2009; Ofuji et al., 2001; Ofuji et al., 1999; Rehmann et al., 2008). Nevertheless, since the first known poisoning event, it took twelve years until the discovery of a primary producer, the dinoflagellate *A. spinosum* (strain 3D9) (Krock et al., 2008; Krock et al., 2009; Tillmann et al., 2009). This small dinoflagellate (12-16 µm length and 7-11 µm width) produces AZA1 and -2 in culture (Tillmann et al., 2009). Since this recent discovery the organism has been encountered in different parts of the world (Ireland (Salas et al., 2011), France (Nezan et Siano, personal communication), Mexico (Hernandez-Becerril et al., 2010), Argentina (Akselman and Negri, 2012)), and AZA occurrences are now recognized as a worldwide phenomenon. Until now, AZAs were purified and isolated from contaminated bivalves, as it has been originally carried out with other marine biotoxins: okadaic acid group toxins, brevetoxins, saxitoxins, yessotoxins, domoic acid, cyclic imines and pectenotoxins (Rundberget et al., 2007). However, severe toxic events are required to obtain pure standards from contaminated bivalves, even though recovery has been improved recently and the number of purification steps required to purify AZAs from complex matrices reduced (Kilcoyne et al., 2012; Perez et al., 2010).

The primary AZA producer is now identified and adapted to culture, furthermore, natural occurring blooms are hard to predict and/or to find; preventing *in situ* direct extraction of AZA as developed by Rundberget et al. (2007). Thus, to avoid AZA1 and -2 scarcities it is important to have a sustainable production of toxins from *A. spinosum* culture for toxicological studies, and for instrument calibration in continuous monitoring programs.

The aim of this study was to evaluate the feasibility of AZA production from *A. spinosum* produced in continuous pilot scale photobioreactor in series. We describe here how dilution rate influences cell concentration as well as toxin production in pilot scale chemostat bioreactors in series and the use of different solid phase extraction procedures to recover AZAs from large volume of *A. spinosum* culture (200L) after tangential flow filtrations.

2. Materials and methods

2.1. Culture conditions and measurement

The strain (3D9) of *Azadinium spinosum* was the source of AZA1 and -2 for the experiment. The algae were produced in two chemostats of 100 L each, operated in series at different dilution rates (0.15, 0.2, 0.25 and 0.3 day^{-1}). Culture medium was a K modified medium (Keller et al. 1987), without NH_4Cl, tris buffer and with Na_2SeO_3 (10^{-8}M).

The photobioreactors were operating using the following conditions : the pH was maintained at 7.9 using CO_2 addition, T = 18°C, a photon flux density of 200 µmol.m^{-2}.s^{-1} on one side of the reactor, and a photoperiod of 16 h of light and 8 h of dark (Jauffrais et al., 2010). A Rushton turbine was homogenizing the algae at 40 rpm. Algae were collected in a harvesting tank (300 L), aerated and maintained at 18°C (figure 1).

A particle counter (Beckman, Multisizer 3 Coulter counter) was used daily to assess cell concentrations (cells.mL^{-1}), average size (µm) and cellular volume (µm^{3}.mL^{-1}). The bioreactors were considered at steady state after a minimum of five days at the same micro-algal concentration (± 5%).

2.2. Intra- and extra-cellular analysis of AZAs

At the different steady states studied daily over a week, triplicate samples of *A. spinosum* were taken from each bioreactor to assess toxin content, the same analyses were carried out from the 300 L harvesting tank before each tangential flow filtration for initial toxin content assessment.

The analytical procedure had been previously optimised(Jauffrais et al., 2012). Briefly, aliquots (10 mL) of *A. spinosum* cultures were collected and centrifuged (2 500 g, 20 min, 4°C) in 15 mL tubes. The supernatant was collected (for extra-cellular toxin content) and the pellet was re-suspended with 0.5 mL of acetone/H_2O (9/1, v/v), transferred to an Eppendorf tube (1.5 mL) and bath sonicated (10 min). After sonication, the aliquot was centrifuged (15 000 g, 10 min, 4°C). The supernatant was transferred to a 5 mL glass tube and gently evaporated under nitrogen on a heating block at 35°C. This process was repeated so that the pellet was extracted three times in total. After evaporation of supernatants, the residue was reconstituted in 1 mL methanol. Subsequently, an aliquot was filtered with NANOSEP MF filter 0.2 µm (PALL) (15 000 g, 3 min, 4°C), and transferred into a HPLC vial with 250 µL insert for analysis.

After centrifugation of algal culture, the supernatant was transferred to a 15 mL glass tube and 5 mL of dichloromethane was added. The mixture was homogenized and centrifuged (2 500 g, 10 min, 4°C). The organic phase was transferred to a 15 mL glass tube and gently evaporated under nitrogen on a heating block at 35°C. The aqueous phase was extracted three times in this manner, and following evaporation, the residue was reconstituted and filtered as above.

2.3. AZAs harvesting procedures

Tangential flow filtration (Sartorius Stedim Biotech, Sortojet Pump with Sartocon Slice and 5x0.1m^{2} Hydrosart Open Channel Microfiltration Cassettes) was applied to separate the algae from the culture medium. Thus 200 L of algal culture were divided into 1 L of algal concentrate (retentate) and almost 200 L of permeate (figure 1).

For toxin extraction from the retentate, the algal concentrate was sonicated (20min in ice, Bioblock Scientific, Vibra-cell 75115), 25 g of activated Diaion HP20 polymeric resin was added, and gently agitated within the algal concentrate over 24 h, on a laboratory shaker (IKALABORTECHNIK, KS125basic). The resin was then washed with 1 L of Milli-Q water (Millipore, Integral 3 system), and placed in a glass column (3 by 60 cm). The toxin was eluted with three volumes of acetone (50 mL) at 1 mL.min^{-1}. The extract was then evaporated using a rota-evaporator (Büchi, Rotavapor R-200) and the residue was reconstituted in 5 mL methanol.

For toxin extraction from the 200L permeate, two procedures were tested:

1. Passive samplers were placed into the permeate as developed by MacKenzie et al. (2004) and Fux *et al.* (2009; 2008). 8 SPATT bags (solid phase adsorption toxin tracking) containing 3 g of activated Diaion® HP20 resin were added into the permeate and gently agitated within a submerged pump over 72 h. The resin was then extracted as above.

2. A submerged pump (20 L.min^{-1}) was placed into the permeate and connected to a column containing 25 g of activated Diaion® HP20 resin over 72 h. The resin was then extracted as above. This procedure was an adaptation of Rundberget et al. (2007) developed for large scale extraction of micro-algal biotoxin *in situ*.

2.4. LC-MS/MS analysis

The samples were analyzed by LC/MS-MS using an Agilent 1100 model coupled to a triple quadrupole mass spectrometer (SCIEX-Applied Biosystems, API 2000) for quantification of AZAs. 5 µL of each sample were injected into the LC-MS/MS, toxins were separated by reversed-phase chromatography with a silica-based column (Hypersil BDS C8 column, size 50*2 mm, 3 µm particle size; Phenomenex) The A and B mobile phases were 100% water and acetonitrile/water (95/5, v/v) respectively, both containing 2 mM ammonium formate and 50 mM formic acid. The BDS-Hypersil column was eluted isocratically at a dilution rate of 250 µL.min^{-1} (75%B) at 20°C for 10 min.

AZAs were quantified by comparison with a series of AZA-1 standard from the NRC. The two most intense product ions were selected with the following transitions: AZA1 m/z 842.5>824.5 and 842.5>672.4, and AZA2 856.5>838.5 and 856.5>672.4

3. Results and discussion

Cell concentration, mean diameter, cellular volume and toxin content remained constant at steady states when the dilution rate changed. However, differences were noticed between reactors in series (R1 and -2). At the different dilution rates studied cell concentrations were equal to 190 000 and 215 000 cells.mL^{-1} in R1 and R2 respectively and results for mean diameter and cellular volume were comparable at all dilution rates but differed between R1 and R2 (table 1). For each steady state studied, AZAs cell quota increased between bioreactor 1 and -2, showing a positive effect of bioreactors in series to enhance AZA cellular content. Interestingly, contrarily to cellular concentration, AZA cell content decreased as dilution rate increased, ranging from 67 to 24 fg.cell^{-1} for R1 and 98 to 63 fg.cell^{-1} for R2. Thus, the cell production increased as dilution rate increased whereas AZA production reached an optimum at 0.25 day^{-1} of 475 ± 17 µg.day^{-1} under the studied conditions.

Batch cultured *A. spinosum* (strain 3D9 or SM2) produced AZA1 and -2, with AZA1 as the predominant AZA and with a cell quota ranging from 5 to 40 fg.cell^{-1} (Jauffrais et al., 2010; Salas et al., 2011; Tillmann et al., 2009). In the present study, the same toxin profiles were found, however, AZAs cell quota of 24 to 98 fg.cell^{-1} were obtained depending on the dilution rate. There was a higher toxin concentration at low growth rate of *A. spinosum* than higher dilution rate (especially in R1), showing the necessity of chemostats in series at higher dilution rate to increase significantly toxin concentration.

As described above, continuous *A. spinosum* culture was shown to be valuable for production of AZAs using photobioreactors in series. Subsequently, AZA extractions were developed to optimise the recovery from bioreactors. Before filtration, 95% of the toxin was intracellular, whereas after filtration, 50 to 70% of the toxin was contained in the concentrate and 30-50% released in the permeate. The observed variation was time dependent, with longer filtration times leading to higher proportions of toxin in the permeate.

Intracellular toxin content was recovered as algal paste after centrifugation of the retentate, however, this procedure inferred the loss of some toxin from the supernatant (± 10%). To avoid this loss a solid phase adsorption was implemented using Diaion® HP20 resin as

explained above, this procedure allowed for the recovery of up to 90% of the total toxin from the retentate.

Extracellular toxin content was extracted using two procedures, the SPATT bags and a solid phase extraction procedure. SPATT bags were initially designed as a monitoring tool to follow and predict micro-algal toxic event around shellfish production areas (MacKenzie et al., 2004; MacKenzie, 2010). The solid phase extraction procedure was implemented for biotoxin extraction from naturally occurring micro-algal blooms (Rundberget et al., 2007). These two methods allowed good recovery, however, recovery using SPATT bags showed more variability than the SPE procedure in the condition tested. Even though, the procedures for AZAs extraction from the concentrate and permeate allowed for the recovery of 80 ± 5% of toxins originally produced by *A. spinosum* pilot scale culture.

4. Conclusion

At a rate of 0.25 day^{-1}, we obtained about 3 mg of AZAs in crude extracts over 12 days (8 days of culture, 1 day of filtration and 3 days of extractions). The optimisation of the procedure demonstrated the feasibility of producing AZAs from *A. spinosum* cultured in photobioreactors in series. The AZAs obtained are suitable amounts for purification and production of certified standards for further toxicological study and for instrument calibration in monitoring programs.

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Table

Table 1. *A. spinosum* concentration (cells.mL^{-1}), mean diameter (µm), cellular volume (µm^3.mL^{-1}), toxin content (fg.cell^{-1}), and cell and toxin productivity (cells.day^{-1} and µg.day^{-1} respectively) at the different dilution rate studied (0.15, 0.2, 0.25, 0.3 day^{-1}) in the two bioreactors in series (R1 and -2).

	0.15 D^{-1}		0.2 D^{-1}		0.25 D^{-1}		0.3 D^{-1}	
	R 1	R 2	R 1	R 2	R 1	R 2	R 1	R 2
***A. spinosum* concentration** (cells. mL^{-1})	193000 ± 6000	214000 ± 3000	194000 ± 8000	214000 ± 7000	190000 ± 6000	221000 ± 5000	187000 ± 5000	220000 ± 4000
***A. spinosum* mean diameter** (µm)	9.59 ± 0.15	9.90 ± 0.16	9.63 ± 0.23	10.11 ± 0.18	9.29 ± 0.09	9.93 ± 0.04	9.48 ± 0.12	10.02 ± 0.05
***A. spinosum* cellular volume** (10^{7}xµm^3.mL^{-1})	9 ± 0.4	11 ± 0.4	9 ±0.6	12 ± 0.7	8 ± 0.3	12 ± 0.2	8 ± 0.1	12 ± 0.4
AZA1 (fg.cell^{-1})	52 ± 6	74 ± 4	34 ± 12	76 ± 14	26 ± 2	61 ± 3	17 ± 1	45 ± 3
AZA2 (fg.cell^{-1})	15 ± 1	24 ± 2	10 ± 2	19 ± 2	12 ± 2	25 ± 2	7 ± 1	18 ± 2
AZAs (fg.cell^{-1})	67 ± 3	98 ± 5	44 ± 13	95 ± 16	38 ± 2	86 ± 3	24 ± 1	63 ± 5
Cell productivity (10^{9}xcells.day^{-1})	2.90 ± 0.09	3.21 ± 0.05	3.90 ± 0.16	4.28 ± 0.14	4.75 ± 0.15	5.53 ± 0.13	5.61 ± 0.15	6.60 ± 0.12
Toxin productivity AZA1+AZA2 (µg.day^{-1})	193 ± 9	314 ± 15	170 ± 50	406 ± 64	180 ± 10	475 ± 17	134 ± 5	415 ± 33

Figure

Figure 1. Azaspiracids production system