Growth and toxin production of *Azadinium spinosum* in batch and continuous culture.

Jauffrais, T.[1], Séchet, V.[1], Herrenknecht, C.[2], Tillman, U.[3], Krock, B.[3], Amzil, Z.[1], Hess, P.[1]

[1]IFREMER, centre de Nantes. Département Environnement Microbiologie Phycotoxine. Rue de l'Ile d'Yeu. 44311 Nantes. France. E-mail : thierry.jauffrais@ifremer.fr, [2]Nantes Atlantique Universités, 1 rue Gaston Veil. 44035 Nantes. France. [3]Alfred Wegener Institute. Am Handelshafen 12. D-27570. Bremerhaven. Germany.

Abstract

Azaspiracids are lipophilic marine biotoxins causing gastrointestinal symptoms similar to DSP toxins. Since 1995, azaspiracids have been encountered in Europe, Africa and more recently in North and South America and Japan. The biological primary producer remained undiscovered during many years and has now been identified as *Azadinium spinosum*. The organism was grown using K modified medium, at 18°C with a PFD of 200 µmol.m^{-2}.s^{-1} and a photoperiod of 16L/8D. Batch cultures were carried out using 75mL and 10L flasks, while continuous cultures were produced in 100L chemostats. Cells were recovered using centrifugation or filtration. Different extraction solvents and procedures as well as evaporation modes were evaluated for yield. Quantitation was carried out using LC-MS-MS. *A. spinosum* had a maximum growth rate of 0.6 d^{-1} with K modified medium, and reached maximum cell concentration of 300000 cells.mL^{-1}. Toxins were mostly intracellular, with 5 to 10% toxin in the culture medium. Analogues detected included AZA1, -2 and the methyl esters of AZA1 and -2, AZA1 being the predominant toxin.

Introduction

Harmful algal blooms might cause severe human illness due to the consumption of bivalves, as microalgae are the principle food for bivalve mollusks. In 1995, contaminated mussels (*Mytilus edulis*) from Killary harbor (Ireland) were consumed in the Netherlands (McMahon and Silke, 1996). The toxic agent caused diarrhea, nausea, vomiting and stomach cramps in consumers, symptoms typical for diarrheic shellfish poisoning (DSP). A new toxin was discovered and was named azaspiracid after structural identification by (Satake *et al.*, 1998). Since then azaspiracids have been encountered in Europe, Africa and more lately in South and North America and Japan (Twiner *et al*, 2010). Recently, the biological source of azaspiracids (AZAs), *Azadinium spinosum* (strain 3D9) a small dinoflagellate, was discovered. This organism produces Azaspiracid-1 and 2 (Krock *et al.*, 2009; Tillmann *et al.*, 2009).

Growth and toxicity of dinoflagellates are dependent of various environmental and nutritional factors. Among various environmental factors, salinity and aeration might affect dinoflagellate growth and toxicity. This early work on *A. spinosum* aims to assess the effect of salinity and aeration on growth and toxicity; to evaluate the cell growth and toxin production in pilot scale chemostats and to assess analytical scale extraction procedures for best yield and suppression of AZA1 and AZA2 methyl esters, two possible artefacts of extraction.

Materials and methods

Evaluation of protocols for extraction of azaspiracid from A. spinosum

Cells were recovered using centrifugation or filtration, different extraction solvents (methanol, acetone, ethanol, acetonitrile, dichloromethane, H_2O) and mixtures thereof as well as evaporation modes were evaluated for yield. Methyl ester formation on AZA1 and -2 was studied using methanol-d_4 for extraction and or reconstitution after evaporation.

Culture condition

A. spinosum (3D9) was grown using K modified medium (Keller *et al.*, 1987), without NH_4Cl and with Na_2SeO_3 (1.72 mg.L^{-1}), at 18°C with a PFD of 200 µmol.m^{-2}.s^{-1} and a photoperiod of 16L/8D, in batch and continuous culture. For experimental purpose, the strain was grown in triplicate at six different salinities (10, 20, 30, 32, 35, 40 psu)

adjusted by dilution with Milli-Q® water or evaporation, in sterile 70mL polystyrene flasks. Salinity was checked with a refractometer. The initial culture cell concentration was 5000 cell.mL^{-1} and the culture was grown until stationary phase for each condition.To evaluate the effect of aeration for a future growth in chemostat, *A. spinosum* was cultured in triplicate in 10 L flat bottomed flasks with and without aeration at 35 psu at the same initial cell concentration.
After adaptation to aeration and agitation the microalgae were inoculated in a 100 L medium scale chemostat to evaluate cell growth and toxin production. The flow rate was 0.15 d^{-1}. When the first bioreactor was at steady state it was connected to a second bioreactor to increase cell concentration and toxicity.

Cell growth
Every day or two 1 mL sample was collected to asses cell concentration with a particle counter. A Gompertz model was applied to follow growth kinetics in batch culture, to determine the maximum growth rate (μ_{max} in d^{-1}), the maximum cell concentration (A expressed as $\ln(X/X_0)$ with X in cells.mL^{-1}) and the latency time (λ in days). The model follows the equation:

$$f(x) = A*\exp(-\exp(\mu_{max}*\exp(1)/A*(\lambda-x)+1))$$

Where x is the time (days).

Extraction procedure used and LC-MS-MS analysis
At the end of the experiment on salinity, every two days for the experiment on aeration and twice a week for the continuous culture, triplicate samples (10 mL) were collected in all flasks and bioreactors for toxin analysis. Samples were centrifuged (2500 g, 20 min, 4°C). The supernatant was discarded and the pellet suspended with 500 µL of acetone 90% and sonicated. After sonication the aliquot were centrifuged (15000 g, 10 min, 4°C). Each supernatant were transferred into a 5 mL glass tube and gently evaporated under nitrogen at 35°C. The pellets were resuspended in 500 µL of acetone 90%, homogenised and centrifuged again. These steps were repeated three times in total. After evaporation of the supernatants, they were reconstituted in 500 µL methanol 90%. Subsequently, the samples were filtered (Whatman nanopore 0.2 µm) at 15000 g, 15 min, 4°C, and transferred into HPLC vials with inserts. The samples were then analysed by LC/MS-MS following method C described in Rehmann *et al.*, (2008).

Results and discussion

Extraction procedure
Less formation of AZA1 and AZA2 methyl esters were measured with centrifugation compared to filtration and with extraction with acetone compared to methanol. Highest azaspiracid yield was obtained with methanol/H_2O and acetone/ H_2O ratio of 100, 90 and 80%, compared to lower ratios and other solvents.
Better recovery was obtained when using 5 mL glass tubes during the evaporation procedure compared to 1.5 mL HPLC vials for the evaporation.AZA1 and 2 methyl esters are believed to be artefacts of extraction due to methanol (figure 1) as they are formed mainly during the extraction and in a lower amount during the reconstitution.

Physiology
Growth of *A. spinosum* was observed between 30 and 40 psu, with a μ_{max} assessed between 0.37 and 0.73 d^{-1}. From 10 to 20 psu all cells died between the beginning and the second day of the experiment. The best cell concentration was obtained at 35 psu and the highest toxicity with 30 and 40 psu (table 1).

Table 1. ***Summary of results from the experiment on salinities***

Salinity (psu)	30	32	35	40
μ_{max} (day^{-1})	0.37	0.52	0.52	0.73
Latency time (days)	1.53	1.65	1.77	4.26
A (cells.mL^{-1})	48 813	55 701	71 379	28 934
AZA1 (fg.cell^{-1})	17.77	16.63	12.47	20.56
AZA2 (fg.cell^{-1})	13.21	12.10	9.22	16.12

A. spinosum had a μ_{max} situated around 0.6 d^{-1} with K modified medium, and reached maximum cell concentration of 200000 to 300000 cells.mL^{-1} with aeration and of 80000±5000 cells.mL^{-1} without aeration, azaspiracid final concentration per cell was also higher with aeration than without aeration, i.e 39±4 and 19±4 fg.cell^{-1}, respectively.
The two pilot scale bioreactors (2*100 L, = R1 and R2) were run in series at 0.15 d^{-1} (10 mL.min^{-1}). At steady state, bioreactor 1 had

194 000 ± 6000 cells.mL^{-1} with a toxicity of 62±1 fg.cell^{-1} and bioreactor 2 had 215000±3000 cells.mL^{-1} with a toxicity of 98±5 fg.cell^{-1}. Chemostats in series increased algal concentration and toxicity in the second bioreactor compared to the first one and gave a stable concentration of toxins along time at steady state.

Conclusion

Medium scale bioreactors in series allowed for the continuous production of toxic *Azadinium spinosum* in large volumes. It can be used as source of AZA1 and -2 for contamination of bivalves, for toxicological studies, and for purification of azaspiracid-1 and 2.

Extraction procedure of azaspiracid from *A. spinosum* with methanol, the solvent currently used for the extraction of lipophilic toxins from bivalves produced two artefacts of extraction. Although previously reported by Rehmann *et al.* (2008), in mussels, these artefacts were not observed in such high concentrations after extraction of mussels. Thus, with *A. spinosum* acetone must be used in place of methanol.

Acknowledgements

This study was carried out under the *Sea Change* strategy with the support of the Marine Institute and the Marine Research Sub-Programme of the National Development Plan 2007–2013, co-financed by the European Regional Development Fund.

References

Keller, M.D., Selvin, R.C., Claus, W. & Guillard, R.R.L. (1987). Journal of Phycology 23: 633-638.

Krock B., Tillmann U., John U. & Cembella A.D. (2009).. Harmful Algae 8: 254-263.

McMahon, T. & Silke, J. (1996).. Harmful Algae news 14.

Rehmann, N., Hess, P. & Quilliam, M.A. (2008). Rapid Communications in Mass Spectrometry 22:549-558.

Satake, M., Ofuji, K., Naoki, H., James, K. J., Furey, A., McMahon, T., Silke, J. & Yasumoto, T. (1998). Journal of the American Chemical Society 120:9967-9968.

Tillmann, U., Elbrachter, M., Krock, B., John U., Cembella, A. (2009).. European Journal of Phycology 44: 63-79.

Twiner, M.J., Hess, P., El-Ladki, R., Butler, S., Doucette, G. (2010), 14th International Conference on Harmful Algae.

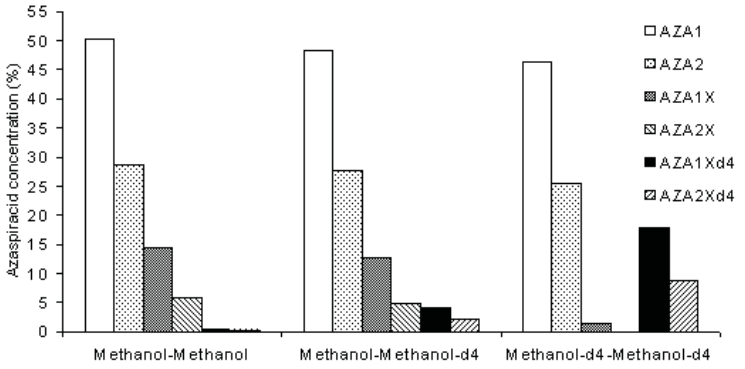

Figure 1. Azaspiracid percentage after extraction and/or reconstitution after evaporation with methanol or methanol-d_4