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Full Length Research Paper

Optimization of laboratory scale production and purification of microcystin-LR from pure cultures of *Microcystis aeruginosa*

Phelan R. R. and Downing T. G.*

Department of Biochemistry and Microbiology, NMMU, P.O. BOX 77000, Nelson Mandela Metropolitan University, Port Elizabeth, 6031, South Africa.

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Microcystin is the most common of the cyanobacterial hepatotoxins with more than 65 known variants. Increased nutrient loading of fresh and coastal water bodies has lead to an increased occurrence of cyanobacterial blooms, many of which are microcystin producing. Due to a limited commercial supply of, and difficulties in obtaining, microcystin standards, there has been an increased interest in production of purified microcystin for the use as standards in analytical laboratories. Microcystin content is however highly variable and optimised culture conditions are essential to produce viable yields of microcystin for purification. We describe the optimization of culture conditions and evaluation of various purification methods to enhance the yield of microcystin from laboratory scale culture.

Key words: Microcystin, purification, standards.

INTRODUCTION

Increased eutrophication of fresh and coastal water bodies has lead to an increased occurrence of toxic cyanobacterial blooms. These blooms are unpleasant and pose a serious health threat due to the presence of toxic metabolites (Oh et al., 2000). Microcystin (MCYST), nodularin and cylindrospermopsin are the most common hepatotoxins (Carmichael, 1994; Harada, 2004). MCYSTs form the largest group with more than 65 variants (Long et al., 2000). MCYSTs are monocyclic heptapeptides with the general chemical structure containing erythro-B-methylaspartic acid (D–MeAsp), alanine (D–Ala), glutamic acid (D–Glu), N-methyldehydroalanine and ADDA, a hydrophobic 20 carbon chain (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6 dienoic acid). Two variable L-amino acids, which differ for the different variants of MCYSTs, are also present as shown in Figure 1. Single letter abbreviations are used in the naming of the toxin in order to indicate the various amino acids that are present in the toxin, i.e. MCYST-LR contains leucine (L) and arginine (R).

*Corresponding author. E-mail: Tim.Downing@nmmu.ac.za.

Intracellular concentrations of MCYST are highly variable in natural environments. Numerous environmental factors have been shown to influence toxin production and investigation of modulation of MCYST production and environmentally affected intracellular concentration has mainly focused on the effects of growth phase (Lee et al., 2000), light intensity (Weidner et al., 2003; Long et al., 2001; Lee et al., 2000; Van der Westhuizen and Eloff, 1985), temperature (Van der Westhuizen and Eloff, 1985; Watanabe and Oishi, 1985), growth rate (Lee et al., 2000; Long et al., 2001; Oh et al., 2000; Orr and Jones, 1998), uptake rates and availability of nitrogen and phosphorus (Lee et al., 2000; Long et al., 2001; Downing, 2005a; Oh et al., 2000; Orr and Jones, 1998; Vezie et al., 2002).

However, Downing et al. (2005b) showed that the primary modulator of cellular MCYST content is the intracellular N : C ratio, with ratios in excess of growth optima yielding increased MCYST content for a given growth rate. Cellular MCYST concentrations are greater under conditions where carbon fixation is limited due to either lack of inorganic carbon or light. This description of culture conditions to enhance MCYST production makes the laboratory scale production of adequate amounts for use as analytical standards feasible.

Lawton et al. (1994), comparing the use of a 100% me-

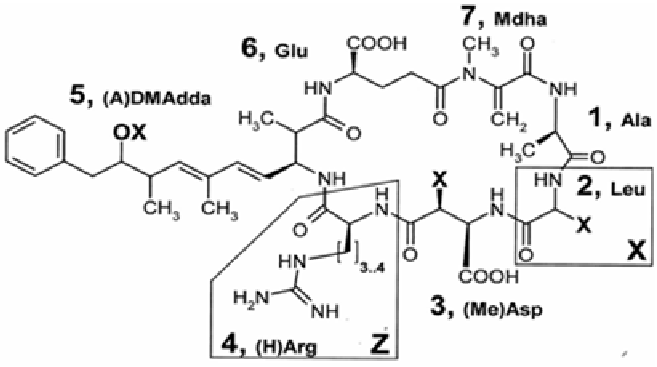

Figure 1. General chemical structure of microcystin. 1 represents D – alanine, 2 and 4 represents variable L-amino acids represented by X and Z, 3 represents D – erythr-B-methylaspartic acid (D – MeAsp) where R can either be CH_3 or H, 5 represents ADDA where x is either $O(CO)CH_3$ or OH, 6 represents D- Glu and 7 represents Mdha (after Lee et al., 2000; Tsuji, 1996; Rapala et al., 2002 and Lawton and Edwards, 2001).

thanol, 75% methanol and water for the extraction of MCYSTs showed that approximately 50% less toxin was recovered when using a 100% methanol compared to either water or 75% methanol. This was confirmed by Ward et al. (1997). Additionally, their results also showed that water and low percentages of methanol showed poor recovery. The highest recovery of MCYSTs was shown with extraction into 50 to 80% methanol, compared to 5% acetic acid, butan-1-ol-methanol-water, water, 100% methanol and percentages of methanol less than 50% (Fastner et al., 1998).

Size exclusion chromatography has generally been used as an initial separation technique to separate pigments from the toxin (Lee and Chou, 2000; Botes et al., 1982). Sephadex LH-20 is the usual stationary phase because it can be used with organic solvents, thus facilitating normal-phase partition chromatography as well as size exclusion (Lawton and Edwards, 2001). Sephadex LH-20 has widely been used for the separation of lipids, fatty acids and small biomolecules. Lee et al. (2000) used a 55 X 3 cm column as an initial clean up step in the separation of seven microcystin variants, where fractions containing MCYSTs were eluted with methanol at a flow rate of 13 ml/min. Toyopearl HW-40F is also frequently used and has a similar mode of action to Sephadex LH-20, (partition chromatography and size exclusion), and has been reported to separate individual MCYSTs with a greater efficiency than Sephadex LH-20 (Lawton and Edwards, 2001).

Lawton and Edwards (2001), Cremer et al. (1991) and Cremer and Henning (1991) suggested the use of ion-exchange chromatography as a preliminary step in the purification of MYCSTs. A quaternary methylamine anion exchange resin such as Pharmacia XK 16/10 column eluted with a mobile phase of 0.2 M ammonium hydrogen carbonate in 30% methanol was used to semi-purify (D-Asp) MYCST variants from methyl variants. Rapid purification of MYCST-LR and nodularin was achieved by Martin et al. 1990 using an unspecified anion resin. A DEAE cellulose column (50 cm x 1.9 cm I.D.) eluted with a mobile phase consisting of a linear salt gradient of 0.005 M to 1 M ammonium hydrogen carbonate was used by Botes et al. (1982) to separate two MYCSTs variants. Anion resins such as quaternary methylamine have been shown to be a very effective as a preparative method for the cleaning up of crude extract and allowing for the rapid purification of MYCST–LR and (Asp^3) MYCST–LR by semi-preparative, reversed-phase HPLC (McElhiney and Lawton, 2005).

Both TLC and high performance analytical TLC (HPTLC) have been used as the final step in purification of MCYSTs typically with silica coated plates and chloroform, methanol and water at various ratios as solvent. The separated samples are visualized using short wave length UV irradiation. Spots that co-elute with known standards are extracted with methanol to remove microcystin and either centrifugation, filtration or both is used to remove the silica (McElhiney and Lawton, 2004). The two main advantages of using TLC for the purify-cation of MCYSTs are complimentary selectivity and low cost. However, a major disadvantage is that it can only be used to separate microgram quantities of microcystin (McElhiney and Lawton, 2004).

Reversed-phase HPLC (RP-HPLC) has been extensively used in the purification of MCYSTs (Rapala et al., 2002). Preparative HPLC has a distinct advantage over all the other procedures that are mentioned above is that it has online detection and there is therefore no need to examine fractions to determine which ones contain the MCYSTs. The required equipment is however expensive and the wide range of column manufacturers and methods must be optimized for each laboratory and column. The majority of stationary phases used for preparative HPLC of MCYSTs are C_{18} bonded to silica (McElhiney and Lawton, 2005).

One of the most frequently used analytical techniques for the analysis of MCYSTs is RP-HPLC in conjunction with photodiode array (PDA). The separation of MCYSTs is highly dependent on the mobile phase that is used. Isocratic mobile phases and gradient elution are both used in the separation of MCYSTs. However, when a gradient has been employed, it has shown a greater efficiency in separating various MCYSTs. The addition of 0.05% trifluoroacetic acid (TFA) to both water and acetonitrile, while using a gradient of acetonitrile (Akin-Oriola and Lawton, 2005) and water has shown excellent separation of 10 variants of microcystin, which differ in polarity. However, it is necessary to compare the results obtained with micro-cystin standards in order to accurately identify the variant of toxin present (McElhiney and Lawton, 2005). Dimethyl variants of MYCST-LR have been successfully separated by using a gradient of ammonium acetate and aceto-nitrile. These variants of

MCYST are normally co-eluted when a gradient of acidified water (0.05% TFA) and acetonitrile is used as a mobile phase. The use of TFA in the mobile phase of preparative systems would appear to be ideal, as it is volatile and a desalting stage is not required. Lawton and Edwards (2001) suggested that TFA has not been widely adopted for preparative HPLC of MYCST, due to problems experienced in the final concentrating step. Prolonged exposure of MYCST to TFA affects the stability of the toxins, leading to the formation of degradation products.

PDA detection coupled to HPLC offers a means of confirming the MCYST peak. However, a shortcoming to PDA detection is that variants of MCYSTs have similar absorption profiles between 200 and 300 nm, limiting the ability to distinguish between the variants. This is because the main chromophore of MCYST is a conjugated diene in the ADDA residue, which absorbs at a wavelength of 238 nm. MCYST-LW can be detected with greater ease using PDA because of its different absorbance spectra due to tryptophan (222 nm) (McElhiney and Lawton, 2005). A major disadvantage of HPLC- PDA detection is the high cost and lack of commercially analytical standards. Thus, a majority of toxins are often compared to purified MCYSTs and the concentrations are reported in terms of MCYST–LR equivalence (Rapala et al., 2002).

HPLC coupled with mass spectrometry is a far more accurate method for the identification of MCYSTs. The information provided by mass spectrometry provides a finger print for the individual toxins and can be used to confirm the presence of various toxins. HPLC-ESI-MS has been used extensively in the detection and identification of various cyanobacterial toxins including MCYSTs. ESI-MS (electro-spray ionization mass spectrometry) produces mainly protonated ($M + H^+$), or divalent ions ($M + 2H^+$), which provide molecular weight information (McElhiney and Lawton, 2005). Thus, allowing for the identification of MCYST variants. ESI-MS can be used to confirm and identify a peak obtained by HPLC-PDA. The data obtained from HPLC-ESI-MS are easy to analyze and is an effective tool for the monitoring of MCYSTs. ESI-MS has the ability to detect picogram quantities of toxin and has been success-fully used in the detection of trace amounts of MCYSTs in cyanobacterial blooms and water samples (Welker et al., 2004).

All of the common analytical methods require reference standards which are difficult to obtain and costly. This research was undertaken to optimize the laboratory scale production and purification of MCYST variants for use as standards for HPLC analysis of potentially contaminated water.

MATERIALS AND METHODS

Optimization of microcystin production

In order to establish optimal culture conditions, 10 ml of *Microcystis aeruginosa* PCC7806 at an OD_{740} reading of 0.752 in BG_{11} (Rippka et al., 1979) was inoculated into Falcon tubes containing either 10 ml of BG_{11} (0.03 mM NH_4^+, 17.65 mM NO_3^-), 10 ml of modified BG_{11} (7.53 mM NH_4^+, 25.15 mM NO_3^-), 10 ml of modified BG_{11} (0 mM NH_4^+, 20.65 mM NO_3^-), 10 ml of modified BG_{11} (0 mM NH_4^+, 23.6 mM NO_3^-), or 10 ml of modified BG_{11} (0 mM NH_4^+, 26.65 mM NO_3^-). All media was at pH 7.4. This was incubated for 96 h under continuous illumi-nation (11.4 µmol/m^2/s, Triton Dayglo©). Cultures were collected by centrifugation at 2500 g for 10 min (Beckman Avanti J-20 centrifuge). The resulting pellet was snap frozen in liquid nitrogen and lyophilized overnight in a VirTis bench top freeze dryer with a condenser temperature of -53.9°C and a vacuum of 350 mTOR. 2 ml 70% methanol was added to freeze dried cultures, which were sonicated in a Bandelin Souvrex NK51 for 10 h. The cell debris was removed by centrifugation at 1000 g for 10 min and the supernatant was collected and desiccated in a speed vac. The dried extract was dissolved in a buffer containing 50 mM Tris-HCl; 0.1 mM EGTA; 0.03% (v/v) Brij35 at a pH 7.5. Toxin concentration was determined using the Abraxis Microcystin/Nodularin Elisa kit (96T).

For laboratory scale production, axenic *M. aeruginosa* PCC7806 was cultured in 500 ml BG_{11} at 23°C (±0.5°C) with constant illumination (11.4 µmol/m^2/s, Triton Dayglo©) and occasional agitation. At an OD_{740nm} of 1.0 the, the culture was transferred to a 10 L bubble reactor, containing an additional 500 ml of BG_{11} and grown under the same light conditions and aeration (<100 ml/min) with filtered air. At an OD_{740nm} of 1.0 another litre of medium was added. This step up procedure was repeated until the total volume of the culture was 4 L at an OD_{740nm} of 1.0. 2 L of sterile modified BG_{11} (7.53 mM NH_4^+, 25.15 mM NO_3^-) was added and grown to an OD_{740nm} of 1.0. Four L of modified BG_{11} (0 mM NH_4^+, 26.65 mM NO_3^-) was added. Aeration was stopped to increase cellular N:C ratio (Downing et al., 2005a) and the culture was grown at 23°C (±0.5°C) with constant illumination (5.3 µmol/m^2/s, Triton Dayglo©) and occasional agitation until an OD_{740nm} of 0.7 was reached.

Optimization of microcystin extraction

Cells were harvested by centrifugation at 10,000 g for 10 min and snap frozen with liquid nitrogen. After lyophilization overnight, 70% methanol was added at 50 µl per mg of dried mass (Downing et al., 2005a). Cell disruption was either by sonication in a Bandelin Souvrex NK51 for 16 h; sonication for one burst of 30 s; pulsing at 70% duty cycle and 40% power on ice using a Bandelin Sonoplus ultrasonic sonicator probe or sonication for eight bursts of three min; pulsing at 50% duty cycle and 40% power on ice using a Bandelin Sonoplus ultrasonic sonicator probe. The cell debris was removed via centrifugation at 2000 g for 10 min and the super-natant desiccated in a speed vac.

Purification of microcystin

A Sephadex – LH20 column (0.5 x 16 cm) was equilibrated with 80% methanol (Lee and Chou, 2000). 3 ml of extract was loaded onto the column and eluted in 80% methanol at a flow rate of 0.5 ml/min. 4 ml fractions were collected and the absorbance at a 238 nm was determined. 238 nm absorption peaks were collected and concentrated using a speed vac and analyzed by either using PPI or Abraxis Microcystins/Nodularins ELISA kit (96T).

A Sephadex – LH20 column (2.5 x 38 cm) was equilibrated with 80% methanol (Lee and Chou, 2000). 15 ml of extract was loaded onto the column and eluted in 80% methanol at a flow rate of 3 ml/min. 7 ml fractions were collected and the absorbance at a 238 nm was determined.

An Anion exchange (Dowex 1-x8, 200 - 400 mesh chloride form) column (0.5 cm x 13 cm) was equilibrated with 0.2 M ammonium hydrogen carbonate. 3 ml of extract was loaded onto the column and eluted in a stepwise gradient from 0.2 M to 1 M ammonium hydrogen carbonate. Two milliliter fractions were collected and the absorbance at 238 nm was determined. Peaks that showed a positive for either PPI inhibition or ELISA were pooled and desic-

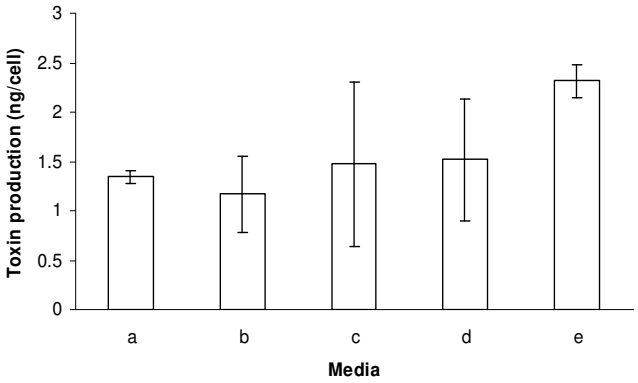

Figure 2. Toxin content after incubation for 96 h in the following media: (a) BG_{11} (0.03 mM NH_4^+, 17.65 mM NO_3^-), (b) modified BG_{11} (7.53 mM NH_4^+, 25.15 mM NO_3^-), (c) modified BG_{11} (0 mM NH_4^+, 20.65 mM NO_3^-), (d) modified BG_{11} (0 mM NH_4^+, 23.65 mM NO_3^-) and (e) modified BG_{11} (0 mM NH_4^+, 26.65 mM NO_3^-). n = 3, error bars are standard deviation of mean.

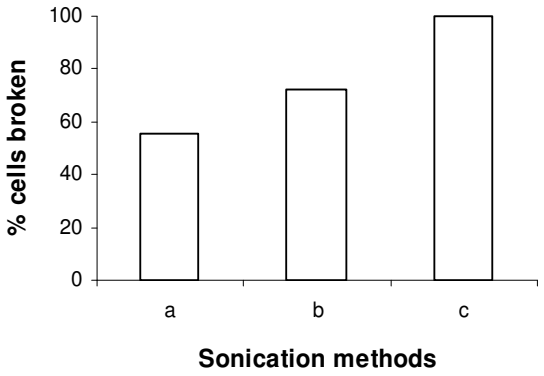

Figure 3. Percentage broken cells after sonication (a) Bandelin Souvrex NK51 water bath sonicator for 16, and with Bandelin Sonoplus ultrasonic sonicator probe (b) one burst of 30 s; pulsing at 70% duty cycle and 40% power on ice, (c) eight bursts of 3 min; pulsing at 50% duty cycle and 40% power on ice.

cated in a speed vac.

Flash chromatography was carried out as per manufacturer's specifications (Waters Oasis® sample extraction products).

Preparative HPLC was used as the final purification step of MCYST extract. The dried toxin was resuspended in 30% methanol and injected onto a C_{18} column (Gemini 5u C_{18} 110A, Axia Packed, 100 x 21.2 mm, Phenomenex) and on the same column and run at a flow rate of three $ml.min^{-1}$. The mobile phases for the HPLC process were methanol and Milli-Q water. The same gradient method for analytical HPLC was used (ISO/FDIS 20719, 2005). Absorption spectrum between 200 and 300 nm was measured using a PDA detector.

Quantification of microcystin

Dried toxin was resuspended in 50 mM Tris-HCl, 0.1 mM EGTA, 0.03% (v/v) Brij35 buffer (pH 7.5). 100 µl of 20 mM p–nitrophenyl

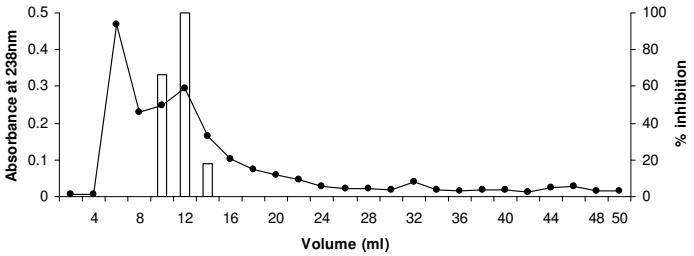

Figure 4. The elution profile obtained with 3 ml crude microcystin extract on Sephadex-LH20 (column 0.5 x 16 cm) eluted at a flow rate of 0.5 ml/min. Fractions from peaks with increased 238 nm absorption were tested with PPi to confirm the presence of toxin

phosphate in 50 mM Tris–HCl; 0.1 mM EGTA (pH 7.5); 0.1% (v/v) 2-mercaptoethanol (pH 7.5) and 50 µl of 20 mM $MnCl_2$ in dH_2O were added to each well of a 96-well microtitre plate. To this, 100 µl toxin extract and 0.5 U protein phosphatase 2A (SIGMA) in 50 µl 50 mM Tris-HCl, 0.1 mM EGTA, 0.03% (v/v) Brij35 buffer (pH 7.5) were added. The absorbance was determined at 410 nm every 3 min for 30 min in a Multiskan microtitre plate reader (Ash et al., 1995; Ward et al., 1997).

Quantification of microcystin by ELISA assay was carried out as per manufacturers' specifications (Abraxis Microcystins/Nodularins ELISA kit, 96T).

Dried toxin was resuspended in 30% methanol and injected onto a C_{18} column (Gemini 5u C_{18} 110A, 250 x 4.6 mm, Phenomenex) for separation and detection as per ISO/FDIS 20719 (2005).

Optimization of microcystin storage

Toxin eluted from preparative HPLC was collected by hand and evaporated under vacuum and resuspended either in 100% ethanol, methanol, 50 mM Tris-HCl, 0.1 mM EGTA, 0.03% (v/v) Brij35 buffer (pH 7.5) or ddH_2O to a concentration of 500 $ng.ml^{-1}$. These were stored at 4°C in the dark and ELISA was used to quantify the toxin weekly for 28 days.

RESULTS

Evaluation of various media compositions, based on previously published data on the role of nitrogen in microcystin production and the effect of carbon fixation on modulating this role, yielded maximum reproducible toxin content with the highest tested level of nitrate and no ammonium (Figure 2).

Figure 3 shows the results of various methods of cell disruption. Sonication in a Bandelin Souvrex NK51 water bath sonicator for 16 h showed the lowest breakage of the cells whereas sonication for one burst of 30 s; pulsing at 70% duty cycle and 40% power on ice using a Bandelin Sonoplus ultrasonic sonicator probe showed an increase in cell breakage. Complete disruption of all the cells was observed with sonication for eight bursts of 3 min; pulsing at 50% duty cycle and 40% power on ice using a Bandelin Sonoplus ultrasonic sonicator probe.

Figure 4 shows the elution profile and PPi inhibition for isolation of microcystins on a small (0.5 x 16 cm) Sephadex-LH20 column. Two major 238 nm peaks were

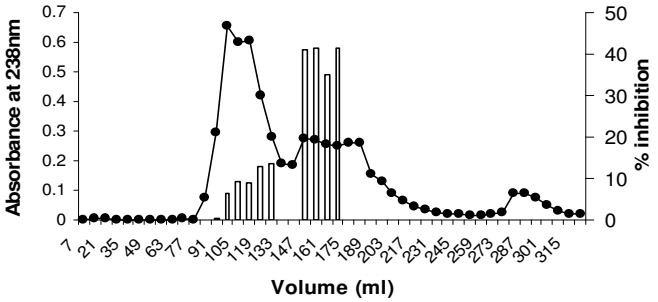

Figure 5. The elution profile from 15 ml of concentrated extract loaded onto a Sephadex-LH20 column (2.5 x 38 cm) eluted at a flow rate of 3 ml/min. Fractions from 238 nm peaks were tested with PPi to confirm the presence of toxin.

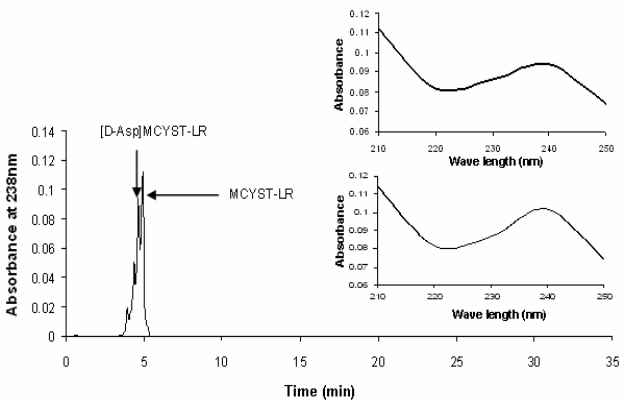

Figure 6. Chromatogram of [D-Asp] MYCST-LR and MYCST-LR extracted from *M. aeruginosa* PCC7806. 10 µl of eluent off the Sephadex-LH20 was injected onto a C_{18} column (Gemini 5u C_{18} 110A, 250 x 4.6 mm, Phenomenex) a flow rate of 0.5 ml/min. The mobile phases for the HPLC process were acetonitrile with 0.05% TFA and Milli-Q water with 0.05%

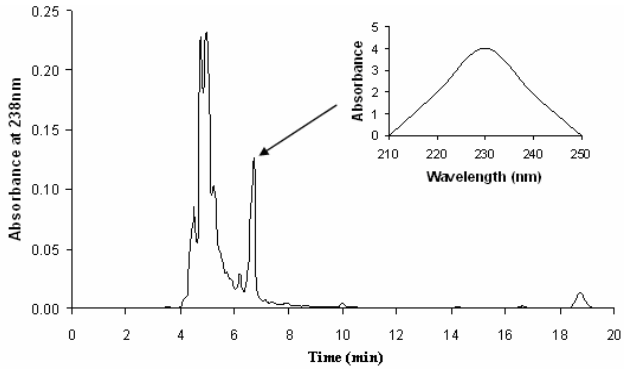

Figure 7. Chromatogram of one milliliter of the waters Oasis® HLB extraction cartridge cleaned up extract was injected onto a C_{18} column (Gemini 5u C_{18} 110A, Axia Packed, 100 x 21.2 mm, Phenomenex) a flow rate of 3 ml/min. The mobile phases for the HPLC process were Methanol and Milli-Q water.

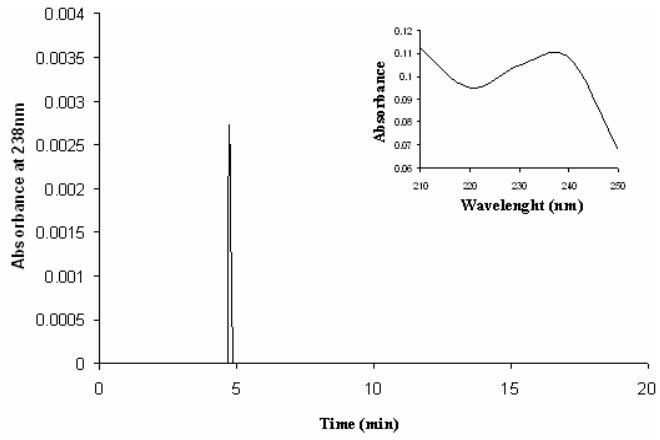

Figure 8. Chromatogram of hand collected peak from preparative HPLC. 10 µl of the pooled cleaned up extract was injected onto a C_{18} column (Gemini 5u C_{18} 110A, 250 x 4.6 mm, Phenomenex) a flow rate of 0.5 ml/min. The mobile phases for the HPLC process were acetonitrile with 0.05% TFA and Milli-Q water with 0.05% TFA.

observed. No PPi inhibition was observed in the first peak. Fractions collected from the second peak inhibited protein phosphatase strongly. Pigments were effectively separated from microcystins, eluting in peaks 3 and 4. This was the desired result because pigments increase the rate of degradation of microcystin in the presence of light. The efficiency of pigment removal prompted scale up for clean-up of larger amounts of crude microcystin extract as shown in Figure 5. With scaled up gel exclusion, a large amount of toxin was present in the first peak indicating a possible maximum loading rate. Most of the toxin eluted in the second peak. As with the smaller column, the pigments were adequately separated from the microcystin.

Reverse-phase HPLC of pooled samples from peak 2 in Figure 6, revealed two major peaks with appropriate absorption spectra. Ion-exchange chromatography of the concentrated pooled samples of peak two in Figure 5 showed one major 238 nm absorption peak suggesting that ion-exchange chromatography was unsuitable to separate the (D-Asp) MYCST-LR from MYCST-LR.

Preparative reverse-phase HPLC of the eluted sample from the Waters Oasis® HLB extraction cartridge showed five peaks (Figure 7). Peak 4 (Figure 7) had maximum absorption spectra of 234 nm. Ten microliters of the hand-collected peak 4 was run on the analytical column and only one peak with appropriate absorption spectra was observed (Figure 8). A positive ELISA result confirmed the presence of toxin in this peak.

A larger percentage of toxin was recovered after the Waters Oasis® HLB extraction cartridge clean-up step of the crude extract compared to the Sephadex LH-20 and concentration step. The total loss in toxin from the complete purification process using size exclusion was 51% compared to the total loss of toxin from complete

Table 1. Percentage toxin recovered after each purification steps.

Process A	Toxin recovery (%)	Process B	Toxin recovery (%)
Waters Oasis® HLB	57	Sephadex LH-20	72
Concentration step (Speed vac)	NA	Concentration step (Speed vac)	41
C_{18} column (preparative)	49	C_{18} column (preparative)	33

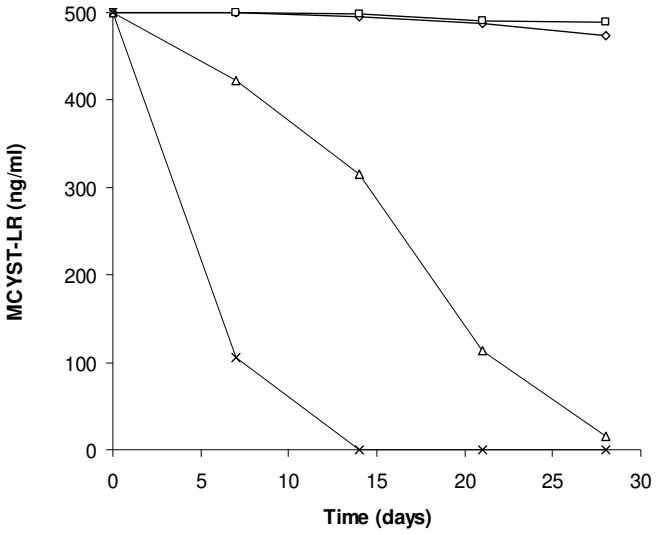

Figure 9. The stability of MCYST-LR under various solvent storage conditions ■ 100% Methanol, ♦ 100% Ethanol, ▲ Buffer 50 mM Tris-HCl, 0.1 mM EGTA, 0.03% (v/v) Brij35 buffer (pH 7.5) and x ddH_2O

purification process using C18 solid phase extraction was 67% (Table 1).

Storage results (Figure 9) showed that minimal degradation occurred when MCYST-LR was stored in either ethanol or methanol. Whereas, complete degradation of the toxin occurred in 28 days when stored in buffer and only 7 days when stored in ddH_2O.

DISCUSSION

Microcystin production

The optimum molar N:P ratio for microcystin production by *M. aeruginosa* PCC7806 was 148:1. This is significantly more than the reported previously reported optimum N:P medium ratio of 46 (Downing et al., 2005) presumably due to the ammonium in the original BG11 which as can be seen from Figure 2, appears to reduce microcystin production. Toxin production was optimal at lower light intensities (5.3 $\mu mol.m^{-2}.s^{-1}$) with no aeration and an increased medium N:P ratio lacking ammonium. This resulted in a MYCST yield of 0.5 $mg.l^{-1}$ at an OD_{740} of 1.0 with PCC 7806.

Microcystin extraction

A longer sonication period with a higher intensity was required to break 100% of the cells. A negative factor of a longer sonication period was that complete breakage of the cells lead to more pigments being extracted. However, this problem was alleviated by use of the Sephadex LH-20 column for pigment removal. Lawton and Edwards (2001) reported that sonication is the most commonly used method of extracting MYCSTs. However, a comparison of published data by Lawton and Edwards (2001) suggests that it has little effect on MYCST yields.

Microcystin purification

Successful clean-up of crude extract was achieved by using a Sephadex LH-20 column (2.5 x 38 cm) as described in the methods. The toxin fraction eluted separately from the pigments. Two disadvantages of the clean-up procedure include a 28% loss of toxin yield and dilution of eluted toxin fraction. Despite several reports of effective separation of variants using ion-exchange , we obtained no separation was obtained using Dowex 1-x8, 200 - 400 mesh chloride form as described in the methods.

Storage of the semi-purified extract is a major hurdle in the purification of milligram quantities of MYCST. Degradation is due to either the presence of contaminants or exposure to light that may either alter or degrade the ADDA ring (Mazur, 2001). Degradation of the toxin occurred either during the desiccation, storage or both after elution from the Sephadex LH-20 column. The long Sephadex LH-20 clean-up step, which dilutes toxin and requires long concentration steps, should be replaced with a more rapid C_{18} clean-up cartridge step. Lawton and Edwards (2001) reported that the use of pre-packed C_{18} clean-up cartridges has facilitated simplistic scale-up of MYCST purification, enabling extracts containing mg quantities of MCYST to be partially purified in a single run. The task of further purification was less complicated due to greatly simplified fractions as well as the concentration of many minor MYCSTs, which were increased in a single step.

Successful purification of a MYCST variant was achieved using two chromatographic steps. The first was using a Waters Oasis® HLB extraction cartridge to remove the pigments and secondly a single preparative HPLC step to obtain purified toxin. 57% of the toxin was recovered after

the Waters Oasis® HLB extraction cartridge was used for clean-up of crude extract. Rapala et al. (2002) reported a similar result with large amounts of toxin being lost using an initial clean-up cartridge step. 49% of the toxin was recovered after preparative HPLC, using gradients of methanol as a mobile phase without TFA. Lawton and Edwards (2001) reported that the presence of TFA affects the stability of the toxin during the final concentrating step.

The results obtained indicated that the clean-up of crude extract by Sephadex-LH20 column was superior in comparison to the Waters Oasis® HLB extraction cartridge due to less toxin being lost after the initial clean-up step. However, the Waters Oasis® HLB extraction cartridge is advantageous as it does not require a concentration step, due to its smaller elution volume. Therefore, the Waters Oasis® HLB extraction cartridge may be a more viable method for the clean-up of crude extract because of the speed of the clean-up and that no concentration step is required.

Conclusion

The optimal procedure for the production and purification of MCYST was as follows: Axenic culture was first grown in modified BG_{11} (7.53 mM NH_4^+, 25.15 mM NO_3^-) to an OD_{740nm} of 1.0 at 23°C (±0.5°C) with constant illumination (11.4 µmol.m^{-2}.s^{-1}, Triton Dayglo©) and gentle aeration (< 100 ml/min) from filtered air. Modified BG_{11} (0 mM NH_4^+, 26.65 mM NO_3^-) was then added and aeration was stopped. The culture was grown at 23°C (±0.5°C), constant illumination (5.3 µmol/m^2/s, Triton Dayglo©) and occasional agitation until an OD_{740nm} of 0.7 was reached. MCYST was extracted in 70% methanol while sonicated at 40% power on ice using a Bandelin Sonoplus ultrasonic sonicator probe. The extract is purified in a two step process: a Waters Oasis® C_{18} extraction cartridge followed by preparative HPLC. This method yielded in excess of 350 µg MCYST-LR per litre of PCC7806 culture. Purified toxin should be stored in 100% methanol.

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