American-Eurasian Journal of Toxicological Sciences 11 (1): 21-28, 2019
ISSN 2079-2050

DOI: 10.5829/idosi.aejts.2019.21.28

Extraction Optimization and Quantification of Chymotrypsin Inhibitors from Cyanobacterium *Microcystis aeruginosa* NIVA Cya43 Using LC/MS

[1]Mohibul Hasan, [2]Kamrul Hasan and [3]Abul Khayer

[1]Department of Environmental Toxicology, Faculty of Biology,
Universität Duisburg-Essen, Universitätsstraße2, 45141Essen, Germany
[2]Department of Bioengineering, Faculty of Graduate School of Natural and
Applied Sciences, Bahçeşehir University, Turkey
[3]Department of Agriculture, Noakhali Science and Technology University,
Noakhali-3814, Bangladesh

Abstract: Cyanobacteria are photosynthetic organisms found in most freshwater and marine system. They are well known for their bloom forming potential. Zooplankton (e. g. Daphnia) can play a important role in controlling cyanobacterial blooms, as they are the major consumers. However, cyanobacteria are a bad food source for Daphnia due to the production of bioactive secondary metabolites. Many cyanobacteria produce these bioactive secondary metabolites that can inhibit the major gut proteases (Trypsin and Chymotrypsin) of Daphnia. Different treatments were tested to determine the best extraction of chymotrypsin inhibitors in *Microcystis aeruginosa* NIVA Cya43. The most efficient extraction was achieved by 80% MeOH use extraction. After extraction, samples were analyzed using liquid chromatography mass spectrometry for quantitative analysis of chymotrypsin inhibitors.

Key words: Extraction Optimization · Chymotrypsin Inhibitors · Cyanobacteria · LC/MS

INTRODUCTION

Cyanobacteria are the oldest life form still present on Earth [1]. They are gram-negative photosynthetic prokaryotes which are found in most freshwater and marine water. For the photosynthesis cyanobacteria evolved a variety of pigments that allow them to harvest a broad light spectrum. Few species of cyanobacteria are capable of fixing atmospheric nitrogen which enables them to access an additional nitrogen source [2]. The most noticeable feature of cyanobacteria is their ability to form blooms in eutrophic surface waters (Lakes, reservoirs and slow rivers) which is mostly caused by an increase in the level of nutrients, usually phosphorus and nitrogen. But other factors like temperature, water column stability can play a role to influence cyanobacterial bloom. In Lake Steilacoom (1994) toxic bloom of *Microcystis aeruginosa* occurred in summer [3]. The characteristics of this lake were associated with higher phosphorus, decreased water transparency, higher water column stability, high surface water temperature and decreased lake flushing [3]. Cyanobacterial blooms lead to water hygienic problems through excretion of volatile compounds which creates unpleasant taste and odor, e.g. geosmin [4]. Freshwater blooms are usually dominated by the cyanobacterial genera *Microcystis*, *Anabaena*, *Oscillatoria*, *Planktothrix* or *Limnothrix* [5].

Herbivorous zooplankton (eg. *Daphnia*) plays an extremely important role in aquatic food chains. They use primary producers as their food source (Algae, cyanobacteria). These zooplanktons are the prey of aquatic insects or many types of small fish, which are themselves eaten by members of higher trophic levels (e.g. bigger fish and humans). Due to the influence on the food change by changes in zooplankton (eg. *Daphnia*)

Corresponding Author: Mohibul Hasan, Department of Environmental Toxicology, Faculty of Biology, Universität Duisburg-Essen, Universitätsstraße 2, 45141 Essen, Germany. Tel: +4917687146240.

populations in aquatic system, affect human need can indirectly be affected. The crustacean *Daphnia* is well studied because of its abundance and important role in aquatic system. *Daphnia* provides an important link for the transfer of carbon and energy from primary producer to higher tropic level [6]. *Daphnia* might also play a role in the control of cyanobacterial blooms because they are unselective filter feeders. They cannot distinguish between good and bad food quality. Cyanobacteria are not a good food source for this crustacean because cyanobacteria lack fatty acids and sterols which is necessary for the growth of *Daphnia* [7]. Some strains of cyanobacteria form filaments (eg. *Anabaena*) that clog the filter apparatus of *Daphnia* [8]. Another reason is the production of secondary metabolites by cyanobacteria [9]. A major focus of recent research is therefore to identify secondary metabolites of cyanobacteria and their detrimental effects on *Daphnia*.

Secondary metabolites are those compounds which are not used by organism for their growth or reproduction [10]. Researchers are interested in these compounds because their effect in still unknown and they might be used in medicine, which has already been shown for some secondary metabolites [11]. *Microcystis aeruginosa* is one of a number of species of cyanobacteria that can produce cyclic peptides, i.e. hepatotoxins, known as microcystins [12]. The cyclic nature of microcystins makes them difficult to be broken down and to be metabolized by animals. Moreover, microcystins block protein phosphatases 1 and 2a by binding irreversible to these enzymes and provoke oxidative stress [13]. Because of growing public health concerns, scientists are working to control the toxicity of blooms of *M. aeruginosa*. The toxic effects of various cyanotoxins have been investigated in mammals and aquatic organisms [14]. In vertebrates, microcystins accumulate in the liver, where it damages liver tissue and promotes tumor growth [15]. Not all strain of *Microcystis aeruginosa* produce microcystins. For example, *Microcystis aeruginosa* NIVA Cya 43 does not produce microcystins [16]. However, they produce a variety of other bioactive metabolites which are cyclic and non-cyclic peptides. They are called depsipeptides with unusual amino acid compositions [16].

An experiment was done to know the transgenerational effect of microcyasin on *Daphnia* [17]. According to their work, it was hypothesized that *Daphnia* are able to withstand microcystin. The concept behind their hypothesis was the biotransformation of microcystin via glutathione S transferase enzyme. During the biotransformation process toxin and toxin conjugate are break down and may form ROS (Reactive oxygen species) which activate several antioxidant enzyme and influence the glutathione-ascorbate cycle. The resulting conjugate are less toxic and hydrophilic for easier excretion.According to their multigenerational experiment enhance tolerance of Daphnia over generation observed [17].

Trypsin and chymotrypsin are the major protease enzymes found in the gut of *Daphnia* [18]. They belong to the group of serine proteases because they cleave peptide bonds in proteins. Cyanobacteria produce some depsipeptides which contains one or more ester bonds in addition to amide bonds. Micropeptolins or cyanopeptolins are one class of cyanobacterial peptides that strongly inhibits trypsin like activity of *Daphnia* [19]. It was found that depsipeptides also inhibit chymotrypsin activity of *Daphnia.* The cyanobacterial strain *Microcystis aeruginosa* NIVA Cya 43 contains two depsipeptides, i.e. BN920 and CP954, which inhibit chymotrypsin activity of *Daphnia* [20]. The same cyanobacterial species but different strain BM25 contains micropeptins (DR1006, DR1056 and MM978) which inhibit chymotrypsin enzymes of *Daphnia magna* [21]. Various studies have been performed in order to identify environmental factors which influence growth rate and toxin production of cyanobacteria. It is found that phosphorus, nitrogen and light have effects on cyanobacterial growth and toxin production [21, 22]. Microcystin production was found to be positively correlated with growth rate under phosphorus limitation [23]. In another study it was shown that photosynthetically active radiation affects the content of microcystin in the *Microcystis aeruginosa* strain PCC7806 [24].

From previous information it is clear that different species of cyanobacteria produce different secondary metabolites, few of these compound interfere digestive proteases (eg. Chymotrypsin) of planktonic crustaceans. The aim of this research work was to develop a method for better extraction of chymotrypsin inhibitors from *Microcystis aeruginosa* NIVA Cya43.

MATERIALS AND METHODS

Preparation of Wright's Cryptophytic (WC) Medium and Population Growth Rate: Normal WC medium was prepared by adding 5 ml of stock solutions A-E and G to 4.5 L ultra pure water and filled up with ultra pure water to 5L.

Table 1: Stock solutions for the preparation of Wright´s Cryptophyte (WC) medium (Guillard 1975)

Stock solution	Ingredient	Stock solution ($g*L^{-1}$)	Medium (mgL^{-1})	Medium (mM)
A	$CaCl_2*2H_2O$	36.8	36.8	0.25
B	$MgS0_4*7H_2O$	37.0	37.0	0.15
C	$NaHCO_3$	12.6	12.6	0.15
D	$K_2HPO_4.*3H_2O$	11.4	11.4	0.05
E	$NaNO_3$	85.0	85.0	1.0
G	Trace elements	Table 2	Table 2	Table 2

Table 2: Composition of stock solution G (Trace elements) for the preparation of WC-medium

Compound	Stock solution ($mg*L^{-1}$)	Medium (mM)
Na_2EDTA	4360	11.7
$FeCl_3*6\ H_20$	3150	11.65
$CuSO_4\ *5H_2O$	10	0.92
$ZnSO_4*7H_2O$	22	0.077
$COCl_2*6H_20$	10	0.042
$MnCl_2*4H_2O$	180	0.91

Microcystis aeruginosa NIVA Cya43 (Collected from Biozentrum Universität köln, Germany) were cultivated in batch culture. For all experiments the cyanobacterium *M. aeruginosa* NIVA Cya 43 was used. Optical density of this pre-cultured cyanobacterial strain were measured at 470 nm with a spectrophotometer (Macherey & Nagel, PF-11). By using Y = 0.198x-0.016 formula volume of pre-culture cyanobacterial cells were measured where X refers optical density and Y refers concentration. Afterwards, medium was inoculated in 300ml conical flasks filled with 100 ml autoclaved WC medium under a sterile bench. The flasks were kept on a horizontal shaker (90 rpm) under constant light condition (46 µmol photons $*m^{-2}*s^{-1}$) at room temperature (20°C).

Cyanobacterial cell abundance was determined daily using light microscopy. Therefore, 0.5 ml subsamples from three randomly choosed flask were taken from each flask under sterile conditions and the cyanobacterial cells were counted by using a Neubauer improved counting chamber (Depth: 0.1 mm; 0.0025 mm^2). To estimate the proper cell abundance at least 100 cells per sample were counted. The experiment lasted 15 days. Based on the cell abundance, population growth rates were calculated in 3 days interval by the following formula.

r [d-1]= @@@@(@@@@@@)−@@@@(@@@@@@)/Δ@@

Formula 1: Population growth rate r of *Microcystis aeruginosa* NIVA Cya 43. Nt1 and Nt2 represent the cell abundances [$Cells*ml^{-1}$] at the time points t1 and t2 and Δt is the time interval between t1 and t2 [d].

Extraction and Quantification of Chymotrypsin Inhibitors from Cyanobacteria

Optimization of the Extraction Method: The chymotrypsin inhibitors have to be extracted from the cyanobacterial cells to measure them using LC-MS. To optimize the extraction process several experiments were performed. First extraction optimization experiments of chymotrypsin inhibitors from *M. aeruginosa* NIVA Cya 43 were done based on different concentration of MeOH use for extraction. These inhibitors were extracted with 40%, 60%, 80% and 100% MeOH and the peak areas of the chymotrypsin inhibitors Nostopeptin 920 and Cyanopeptolin 954 were compared. For the experiment 80% MeOH was used for extraction of inhibitors. Second extraction optimization method was done based on the effect of different duration of extraction time like 0 hour (Immediately), 2 hour and overnight. There was no significant difference was found. For this reason samples were extracted immediately in further experiments. Third extraction optimization method was done to check the extraction from filters is more or less efficient than the extraction from cell pellet. For all treatment NIVA suspension with 20 mg $C*L^{-1}$ (25ml=0.5mg) was used. Total 9 filters with 0.5mg C and 3 filters with 0.3 mg C were prepared. Treatments were labeled with Control, 10ml, soni, freez, ON and 0.3mgC. After prepared filters with cell palate 5ml 100% MeOH was added except treatment '10ml'. In 10ml treatment 10ml 100% MeOH added directly and kept in freeze for 15 minutes. After that 15 minutes sonication (Bandelin, Sonorex Super RK225H) was done except treatment 'Freeze'. In treatment 'Freeze' filters were kept in fridge overnight before sonificate (Bandelin, Sonorex Super RK225H) again. Additional 5ml 100% MeOH was added on the filters and centrifuged at 11.000 rpm for 3 minutes (Eppendorf Centrifuge 5804, Rotor: Eppendorf F34-6-38) and transferred the supernatant in glass tubes. Then supernatant was dried in speed vac (Christ, RVC 2-25) and added 1ml 100% MeOH and transfer to eppendorf tube and dried in speed vac (Christ, RVC 2-25) again. After that 100µl MeOH (100%) added

and transferred in vials for quantification of chymotrypsin inhibitors. Compare to control extraction treatment more or less no differences observed.

Extraction of Chymotrypsin Inhibitors from Cyanobacteria Using Optimized Protocol: Chymotrypsin inhibitors were extracted from *Microcystis aeruginosa* NIVA Cya 43 cells by 80% methanolic extraction. Every third day of experiment, flask with *Microcystis aeruginosa* NIVA Cya 43 were taken from the shaker and number of cyanobacterial cells were counted. The volume of the cultured samples were calculated depending on the cell abundance by using y = 8.9811 x + 16.158 formula, where X refers concentration and Y refers cell abundance. To remain constant of carbon amount (0.25mg) required volume were calculated. The calculated volume was centrifuged at 11.000 rpm for 3 min- (Eppendorf Centrifuge 5804, Rotor: Eppendorf F34-6-38). The supernatant was discarded and the cell pellet was. 10 ml 80% MeOH and 10µl internal standard (MC-LR, concentration was 10 µg*L-1) were added to the cell pellet. After 5 minutes of sonification (Bandelin, Sonorex Super RK225H) this suspension was centrifuged and the supernatant was transferred to a glass tube. The sample was evaporated to dryness by using a vacuum centrifuge (Christ, RVC 2-25). The residue was dissolved in 1ml MeOH (100%) and transferred to an Eppendorf tube and again evaporated to dryness. The residue was re-dissolved with 100µl MeOH (100%) and transferred to glass vials for quantification of the two chymotrypsin inhibitors nostopeptin 920 and cyanopeptolin 954 by using LC- MS.

Quantification of Chymotrypsin Inhibitors by Using LC-MS: The quantification of chymotrypsin inhibitors (Fig. 4) from *M. aeruginosa* NIVA Cya 43 was performed using an Accela ultra high pressure liquid chromatography system (UHPLC, Thermo Fisher) combined with an Exactive Orbitrap mass spectrometer (Thermo Fisher). The chromatography system consists of a 1250 psi pump, an autosampler and a photo diode array detector (PDA). A C18-nucleosil column (EC 125/2 Nucleosil, 100-3; Macherey and Nagel, Düren, Germany) was used as stationary phase. The mobile phase was a gradient (Table 3) of ultra pure water and acetonitrile (ACN) both containing 0.05 % trifluoracetic acid (TFA). The mass spectrometer was run in electrospray ionization mode (ESI) with positive ionization at 325°C and a constant N2 gas flow. Capillary voltage was set to 60 V and the spray voltage was 4.5 kV. For the measurements a scan range between150 to 1500 Da was applied. Xcalibur software (Thermo Fisher) was used for quantitative analyses.

Table 3: Applied solvent gradient for the quantification of chymotrypsin inhibitors (BN920 and CP954) by using internal standard MC-LR.

Time [min]	Solvent A ACN + 0.05 TFA [%]	Solvent B Water + 0.05 TFA [%]
0	38	62
2	40	60
12	50	50
12.5	100	0
15	100	0
15.5	38	62
17	38	62

For each run, 10µl of a sample were injected and sample were measured twice (Technical replicates). Under the applied conditions, BN920 is elutes after 1.85 min and CP954 after 2.21 min (Fig. 1). Based on the measured peak areas chymotrypsin inhibitors in the sample were measured by using calibration curve. The calibration curves were established by Jasmin Jülich (Master Thesis, 2014) using purified chymotrypsin inhibitors and the internal standard MC-LR. The calibration curve for BN920 ranged from 0 to 35 $\mu g*ml^{-1}$ ($y = 0.661777x - 0.207$, $R^2 = 0.9908$) and the calibration curve for CP954 is in the range of 0 to 150 $\mu g*ml^{-1}$ ($y = 0.662427x + 3.66638$, $R^2 = 0.9621$).

Statistical Analysis: Statistical analyses were conducted with the program Sigmaplot 11.0. The data were analyzed using one way ANOVA and a post hoc analysis [Tukey's honestly significant difference (HSD)].Graph were made with Microsoft office Excel 2007.

RESULTS AND DISCUSSIONS

With the aim of optimizing the extraction method identical pellets of *Microcystin aeruginosa* NIVA Cya43 were extracted with 40%, 60%, 80% and 100% methanol (MeOH) after the addition of microcystin-LR as internal standards (Fig. 1). The samples were measured with LC-MS and the peak areas of the chymotrypsin inhibitors CP954 and BN920 were compared to determine the best MeOH concentration. There was no significant difference between the different MeOH concentrations (One way ANOVA, $p_{BN920} = 0.099$, $p_{CP954} = 0.217$). Since 40% and 60% MeOH required more time for the evaporation during the sample preparation, subsequently 80% MeOH was used for the extraction of chymotrypsin inhibitors from this cyanobacterial strain.

After the above described optimization of the extraction solvent, 80% MeOH was used with the aim to compare the effect of differences in extraction of time. Three different extraction times were tested: 0 hour (Immediately), 2 hours and overnight (Around ~18 hours) (Fig. 2). No statistical difference between the different

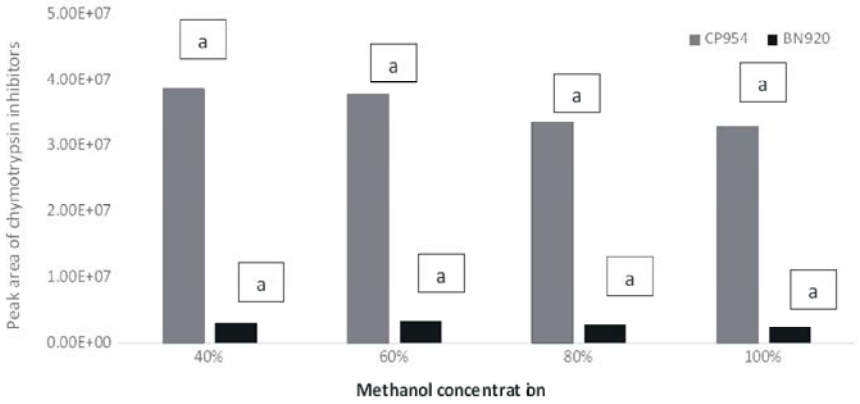

Fig. 1: Effects of different concentrations of methanol on the extraction of the two chymotrypsin inhibitors CP954 and BN920 from *M. aeruginosa* NIVA Cya 43. Depicted are mean values ± SD, n=3. Statistical analysis showed no differences between the treatments (One way ANOVA, $p_{BN920} = 0.099$, $p_{CP954} = 0.217$).

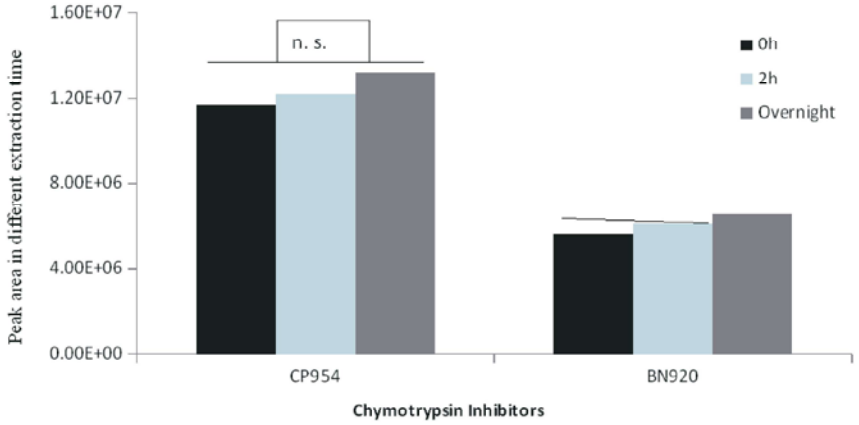

Fig. 2: Effect of different extraction times on obtained peak areas for the two chymotrypsin inhibitor CP954 and BN920 after extraction of *M. aeruginosa* strain NIVA Cya 43 with 80% MeOH. Depicted are mean values ± SD, n = 3. Statistical analysis showed no differences between the treatments (One way ANOVA, $p_{BN920} = 0.889$ $p_{CP954} = p = 0.922$)

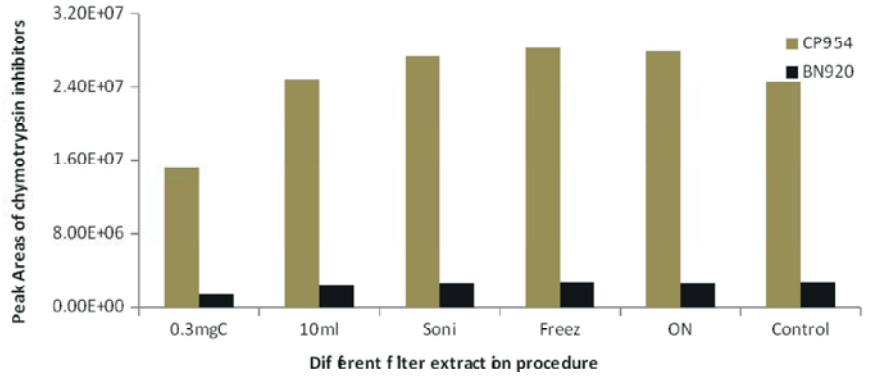

Fig. 3: Effects of different filter extraction procedures on the obtained peak areas for the two inhibitors CP954 and BN920. Depicted are mean values ±SD, n = 3. Different letters indicate a significant differences (Tukey's HSD after one way ANOVA, $p<0.05$). Here, control=normal extraction protocol, 10ml=Extraction from filter with 10ml 100% MeOH, soni=2 times 5ml added to the filters, Freez = keep sample in fridge for 15 munities. ON=Overnight extraction, 0.3mg=Extraction with 0.3 mg C instead of 0.5 mg C

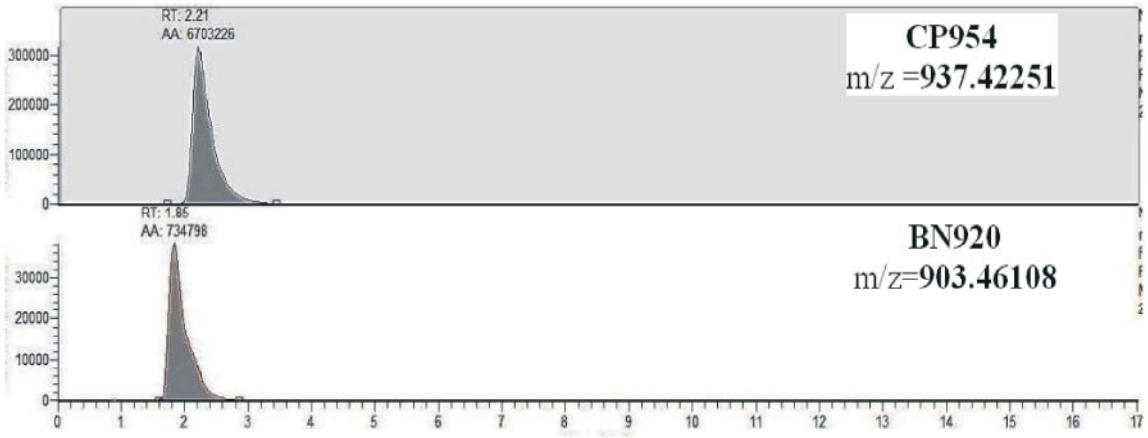

Fig. 4: Example for mass chromatograms of the chymotrypsin inhibitors cyanopeptolin 954 and nostopeptin 920 using LC-MS. Shown are chromatograms for the inhibitors BN920 (m = 903.46108, z = 1, retention time = RT = 1.85 and CP954 (m = 937.42251, z = 1, retention time = RT = 2.21). The injection volume of the experimental sample was 10µl.

extraction times, neither for BN920 (One way ANOVA, p = 0.889) nor for CP954 (One way ANOVA, p = 0.922). Because no difference was found, samples were extracted immediately in further experiments [26-28].

Another extraction optimization experiment was done to investigate the efficiency of the extraction from filters compared to the extraction from cell pellets (Fig. 3). None of the tested procedures with filters showed an increased recovery of the two inhibitors compared to the extraction from cell pellets (One way ANOVA, p_{BN920} = 0.016, p_{CP954} = 0.017).Because of the higher effort for extracting the inhibitors from the filters, the extraction for the experiments was done with cell pellets [29, 30].

Another research work done based on extraction optimization of secondary metabolites (Hepatotoxin microcystin LR) of cyanobacteria. The cyanobacterium *Microcystis aeruginosa* NPLJ-4 was investigated for this study and eight different treatments (a) methanol b) acetic acid c) microwave d) methanol+TFA e) sonic disruption + MeOH f) N_2g) cell disruption h) sonic disruption+ water) were tested to determine the best microcystin LR extraction [31]. The best extraction was achieved by sonicating samples diluted in water because of polar characteristics of microcystin LR.

CONCLUSION

Different treatments were tested to determine the most effective chymotrypsin inhibitors extraction method from cyanobacteria. Although statistical analysis did not show significant differences among the treatments using 40%, 60%, 80% and 100% concentration of methanol (MeOH) but 40% and 60% MeOH required more time for the evaporation during the sample preparation. Because of that, 80% MeOH considered for the better extraction.

REFERENCES

1. Schopf, J.W. and B.M. Packer, 1987. Early Archean (3.3-billion to 3.5-billion-year-old) microfossils from Warrawoona Group, Australia. Science, 237: 70-73.
2. Gallon, J.R., 1992. Reconciling the incompatible: N_2 fixation and O_2. New phytologist, 122: 571-609.
3. Jean, M., C. Jacoby, B. Collier, F. Welch, Joan Hardy and M. Crayton, 2011. Environmental factors associated with a toxic bloom of *Microcystis aeruginosa*. Canadian Journal of Fisheries and Aquatic Science, 57: 231-240.
4. Steven, G., J. Jiang, P. Christopher, E. Saint David, Cane and T. Monis, 2008. Isolation and Characterization of the Gene Associated with Geosmin Production in Cyanobacteria. Environ. Sci. Technol., 42(21): 8027-8032.
5. Martin, T., Dokulil and K. Teubner, 2000. Cyanobacterial dominance in lakes. Hydrobiologia, 438: 1-12.
6. Jonas, P., T. Michael, Brett, T. Vrede, Joseph and L. Ravet, 2007. Food quantity and quality regulation of trophic transfer between primary producers and a keystone grazer (Daphnia) in pelagic freshwater food webs. Synthesizing Ecology, 116: 1152-1163.
7. Dominik, M. and E. Elert, 2009. Good food versus bad food: the role of sterols and polyunsaturated fatty acids in determining growth and reproduction of Daphnia magna. Aquatic Ecology, 4: 943-950.

8. Porter, K.G. and R. McDonough, 1984. The energetic cost of response to blue-green algal filaments by cladocerans. In Limnol. Oceangr, 29(2): 365-369.
9. Orlando, S., S. Gustafsson and L. Hansson, 2010. Effects of cyanobacteria on fitness components of the herbivore Daphnia. Journal of Plankton Research, 32: 471-477.
10. Daniela, R., D. Figueiredo, M. Ulisses and J. Pereira, 2004. Microcystin-producing blooms a serious global public health issue. Ecotoxicology and Environmental Safety, 59: 151-163.
11. Rajesh, P., R. Rastogi and P. Sinha, 2009. Biotechnological and industrial significance of cyanobacterial secondary metabolites. Biotechnology Advances, 27: 521-539
12. Scott, M., J. Brittain, L. Wang, W. Babcock-Jackson, L. Carmichael, Kenneth Rinehart and A. Culver, 2000. Isolation and Characterization of Microcystins, Cyclic Heptapeptide Hepatotoxins from a Lake Erie Strain of *Microcystis aeruginosa*. Journal of Great Lakes Research, 26: 241-249.
13. MacKintosh, C., K.A. Beattie, S. Klumpp, P. Cohen, G.A. Codd, 1990. Cyanobacterial microcystin-LR is a potent and specific inhibitor of protein phosphatases 1 and 2A from both mammals and higher plants. FEBS Lett, 264(2): 187-92.
14. Aloysio, S., Ferrao-Filho and B. Kozlowsky-Suzuki, 2011. Cyanotoxins: Bioaccumulation and Effects on Aquatic Animals. Mar. Drugs, 9(12): 2729-2772.
15. Andrew, R., I. Humpage and R. Falconer, 1999. Microcystin-LR and liver tumor promotion: Effects on cytokinesis, ploidy and apoptosis in cultured hepatocytes. Environmental Toxicology, 14: 61-75.
16. Eric, E., L. Oberer, P. Merkel, T. Huhn and F. Blom, 2005. Cyanopeptolin 954, a Chlorine-Containing Chymotrypsin Inhibitor of *Microcystis aeruginosa* NIVA Cya 43. J. Nat. Prod, 68: 1324-1327.
17. Rafael, O.R., T.S. Dao and C. Wiegand, 2015. Transgenerational effects of microcystin-LR on Daphnia Magna. Journal of Experimental Biology, pp: 2795-2805.
18. Eric, E., M.K. Agrawal, C. Gebauer, H. Jaensch, U. Bauer and A. Zitt, 2003. Protease activity in gut of Daphnia magna: evidence for trypsin and chymotrypsin enzymes. Science Direct, 137: 287-296.
19. Czarnecki, O., M. Henning, I. Lippert and M. Welker, 2006. Identification of peptide metabolites of Microcystis (Cyanobacteria) that inhibit trypsin-like activity in planktonic herbivorous Daphnia (Cladocera). Environ. Microbiol., 8(1): 77-87.
20. Eric, E., A. Zitt and A. Schwarzenberger, 2015. Inducible tolerance to dietary protease inhibitors in Daphnia Magna. Journal of Experimental Biology, 215: 2051-2059.
21. Philip, T. and J. Jones, 1998. Relationship between microcystin production and cell division rates in nitrogen-limited *Microcystis aeruginosa* cultures. Limnol Oceanogr, 43(7): 1604-1614.
22. Schwarzenberger, A., T. Sadler and E. Elert, 2013. Effect of nutrient limitation of cyanobacteria on protease inhibitor production and fitness of Daphnia magna. The Journal of Experimental Biology, pp: 3649-3655.
23. Downing, T.G., C. Meyer, M.M. Gehringer and M. Van de Venter, 2005. Microcystin content of *Microcystis aeruginosa* is modulated by nitrogen uptake rate relative to specific growth rate or carbon fixation rate. Environ toxicol., 20 (3): 257-62.
24. Claudia, W., M. Petra, V.J. Fastner, S. James, M. Geoffrey and L.R. Mur, 2003. Effects of Light on the Microcystin Content of *Microcystis* Strain PCC 7806. Applied and Environmental Microbiology, 69: 1475-1481.
25. Kamal, A.H.M., S. Ghosh, M.S. Hossain and A. Khayer, 2019. Effects of different postharvest treatments on ripening and quality of banana (*Musa sapientum*). International Journal of Research and Review. DOI-10.4444/ijrr.1002/988, 6(2): 303-313.
26. Mian, M.S., M.M. Hasan, A. Khayer and M.A. Habib, 2019. Effects on the Growth Performance and Survival Rate of *Pangasius hypophthalamus* in Different Feeding Rate of Complete Diet. Middle East Journal of Scientific Research. DOI-10.5829/idosi.mejsr.2019.39.54., 27(1): 39-54.
27. Mohsin, A.B.M., F. Yeasmin, S.M. Galib, B. Alam and S.M.M. Haque, 2014. Fish fauna of the Andharmanik River in Patuakhali, Bangladesh. Middle-East Journal of Scientific Research, 21(5): 802-807.
28. Hossain, S., S. Bhowmik, M.T. Hasan, M.S. Islam and M.A. Hossain, 2015. Socio economic condition of Jatka fishers in same selected spots of Meghna estuary. Middle East Journal of Scientific Research, 23(3): 378-386.
29. Mondal, S., M. Rahman, D. Saha, R. Adhikary and M. B. Hossain, 2013. Present status of good aquaculture practices (GAP) in shrimp farms of South-Western coastal area, Bangladesh. Middle-East Journal of Scientific Research, 14(6): 873-878.

30. Ali, M.M., M.B. Hossain, M.H. Minar, S. Rahman and M.S. Islam, 2014. Socio-Economic Aspects of the Fishermen of Lohalia River, Bangladesh. Middle-East Journal of Scientific Research, 19 (2): 191-195.

31. Azim, M.A., M.R. Islam, M.B. Hossain and M.H. Minar, 2012. Seasonal variations in the proximate composition of *Gangetic sillago*, *Sillaginopsis panijus* (Perciformes: Sillaginidae). Middle-East Journal of Scientific Research, 11(5): 559-562.