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Proteomic Profile of the Toxic Cyanobacteria *Microcystic aeruginosa*

RJ Krista Raye Y. Leocadio

Department of Environmental Science and Technology, College of Science and Mathematics, University of Science and Technology of Southern Philippines, Cagayan de Oro, Philippines

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Abstract

The production of cyanotoxins in algal blooms has been a major concern for the quality of freshwaters that aquatic species and humans have in contact with. In particular, microcystin is a common yet potent cyanotoxin that is produced by cyanobacteria. In this paper, the *mcyE* proteome was studied to analyze and gain further knowledge of its role in microcystin production relative to its structure and function. The *mcyE* gene of the *Microcystic aeruginosa* was analyzed using the CyanoOmicsDB database. Based on the findings of data mining, the structure of the microcystin is composed of arginine and leucine. The proteins expressed by this gene cluster are diverse with functions relating to the cell's organization and assembly of complex polyketides or non-ribosomal polypeptides. However, there is little information as to the association of these proteins to the production of microcystin.

* **Corresponding Author:** RJ Krista Raye Y. Leocadio ✉ rjkristaraye.leocadio@ustp.edu.ph

Introduction

Cyanobacteria, also called blue-green algae, belong to the Kingdom Bacteria (Ruggiero *et al.*, 2014). This phylum has five (5) orders, namely, Chamaesiphonales, Chroococcales, Nostocales, Pleuorocapsales, and Stigonematales. These are photosynthetic microorganisms that have successfully survived over three (3) billion years ago on Earth. Importantly, these species have known to contribute to the global biogeochemical cycles. Globally, they are primary producers and greatly provide to the global nitrogen budget (Karl *et al.*, 2002). They act by combining CO_2 and N_2, which is one of the most essential biogeochemical processes on Earth. They also have a role in the Earth's past and present ecosystems (Stal, 2007). Stal (2007) found clues explaining the evolutionary and ecological success of cyanobacteria. Study insights include that these species successfully illuminated almost any environment on Earth, many of which are considered to be antagonistic to life. Somehow, cyanobacteria have a prominent part in many extreme environments including polluted ones.

Over the past two centuries, human-made activities have greatly affected aquatic ecosystems that can over enrich the nutrients (eutrophication), alter the water quality, add up to global warming and contribute to ocean acidification. As a persistent environmental problem, agricultural and industrial activities have been known to cause an over-enrichment of nutrients in freshwater and marine ecosystems, leading to the occurrence of blooms of prokaryotic cyanobacterial species.

The orders Chroococcales, Oscillatoriales, or Nostocales were identified to be involved in nutrient blooms and their species were known as potential producers of cyanobacterial toxins (Codd *et al.*, 2010). Several studies have also claimed that a fraction of 25 to 75% of cyanobacterial blooms is toxic (Bláhová *et al.*, 2007; Bláhová *et al.,* 2008; Chorus, 2001). Of the 150 described genera with 2,600 cyanobacterial species identified, 40 genera are known as toxic (Borges *et al.*, 2015). Most of the studied toxic cyanobacterial species belong to the genera *Dolichospermum*, *Microcystis*, *Aphanizomenon*, *Oscillatoria*, *Aphanocapsa*, *Nostoc*, *Planktothrix,* and *Raphidiopsis*. These species are common constituents of freshwater harmful algal blooms. Swallowing contaminated water or eating contaminated foods with toxins, breathing in aerosolized toxins, and direct contact with contaminated water when swimming or boating are some ways we can be exposed to harmful algal blooms. In the report of the Centers for Disease Control and Prevention (CDC), these species produce cyanotoxins which are known to be the most powerful natural poisons. This led to the limited use of surface waters for irrigation, drinking water, and recreational purposes due to the presence of cyanobacterial toxins. One group of cyanotoxins, the microcystins, is a notable family of more than sixty (65) cyclic heptapeptides produced by some species belonging to the genera *Microcystis*, *Anabaena*, *Nostoc*, and *Oscillatoria* (Rinehart *et al.*, 1994; Sivonen *et al.*, 1996). As seen in Figure 1, this potent liver toxin and possible human carcinogen have been found with eukaryotic serine/threonine protein phosphatases (PP) 1 and 2A inhibitors that share the common structure cyclo(Adda-D-Glu-MdhaD-Ala-L-X-D-MeAsp-L-Z-), where X and Z are variable L-amino acids, Adda is 3-amino-9-methoxy-2,6,8,-trimethyl-10-phenyl-4,6-decadienoic acid, D-MeAsp is 3-methylas partic acid, and Mdha is N-methyl-dehydroalanine (Honkanen *et al.*, 1990). Its mechanism of toxicity is facilitated by the active transport of microcystin into hepatocytes by the bile acid organic anion transport system (Eriksson *et al.*, 1990). Its acute effects can lead to death caused by massive hepatic hemorrhage.

Research studies have proposed a mixed polyketide synthase (PKS)/nonribosomal peptide synthetase (NRPS) origin for the microcystins (Dittmann *et al.*, 1997). In the biosynthesis of microcystins, it was found that the NRPS gene cluster is involved. In the cluster, there are five (5) domains (*mcyABC*) that were sequenced from *Microcystis* strain K139 (Nishizawa *et al.*, 1999). Structurally, the polyketides

and non-ribosomal peptides may be unrelated but are assembled similarly. The metabolites are biosynthesized by coordinated clusters of enzymatic sites called modules, which function in the polyketide or polypeptide chain elongation (von Doëhren *et al.*, 1999). The number and type of catalytic domains in each module describe the structure of the polyketide or peptide structure. Figure 2 shows the *mcy* gene cluster and flanking regions isolated from *M. aeruginosa* PCC7806 with a total of 63.6 kb (Neilan *et al.*, 1997). According to the sequence analysis, the *mcy* region shows a bidirectional operonic structure.

This paper investigated the proteomic profile of *Microcystic aeruginosa* containing the *mcyE* toxic gene using the CyanoOmicsDB database (Zhou, 2021) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa *et al.*, 2000). The choice of the *mcyE* gene is based on research as the most common gene expressed by most toxic cyanobacteria species. In laboratory and field samples conducted by Ngwa *et al.* (2014), *mcyE* gene copies were mostly detected with measurable microcystin levels. Thus, it is best to study the protein profiles of the cyanobacteria as these species produce various toxins. It is important to gain knowledge about the complexity of the biosynthetic pathway to provide information on the functional role of protein metabolites in the environment and the factors that control their production. This, in turn, will promote the control and prevention of the occurrence of harmful algal blooms.

Materials and methods

In the analysis of the microcystin toxin structure, the database from KEGG was used. To investigate the proteomic profile of *Microcystic aeruginosa* and other homologous cyanobacteria species containing the *mcyE* toxic gene, the CyanoOmicsDB (http://www.cyanoomics.cn/) and KEGG (https://www.genome.jp/kegg/) was used to collect the information of the cyanobacterial gene. To date, this database has 8,335,261 entries of cyanobacterial genes from 928 genomes. It can provide multiple gene identifiers, genomic locations, and DNA sequences. Also, the database can predict gene function, amino acid sequences, homologs, and protein-domain superfamilies with accession numbers when studying protein-coding genes. The database has 23,689 gene transcriptional start sites, 96,644 identified peptides, and 16,778 post-translational modification sites retrieved from transcriptomes or proteomes of some cyanobacterial species model. As one of the most common bloom-forming cyanobacteria in freshwater ecosystems worldwide, the *Microcystic aeruginosa* cyanobacteria species was used as model species in this paper. Out of random selection, the strain of the species, gene type, and protein functions of the *Microcystic aeruginosa* NIES-298 were described along with the homologs available in the CyanoOmicsDB database.

Results and discussion

As a model for the toxic cyanobacteria species, the non-ribosomal peptide structure of microcystin-LR was found in KEGG (Fig. 3A). As described by Meyer *et al.* (2016), the common structure of the microcystin heptapeptide is cyclo (D-Ala1 - Xxx^{2} -D-$MeAsp^{3}$ -Zzz^{4} -$Adda^{5}$ -D-Glu6 -$Mdha^{7}$), where Xxx and Zzz represent highly variable L-amino acids at positions 2 and 4 of the cyclic peptide, D-$MeAsp^{3}$ is D-erythro-β-methyl-aspartic acid, $Mdha^{7}$ is N-methyldehydroalanine, and $Adda^{5}$ is 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid. Also, there exists a desmethyl derivative bearing D-Asp^{3}. Singh *et al.* (2016) have described the chemical structure of microcystin-LR (Fig. 3B) where two amino acids, arginine, and leucine, are occupying.

Table 1 presents the result of the *mcyE* gene query in CyanoOmicsDB. There are fourteen (14) species that have been found with the *mcyE* toxic gene.

These species include strains that are identified as *Microcystis aeruginosa* (7), *Microcystis viridis* (1), *Anabaena sp.* (1), *Planktothrix sp.* (1), *Planktothrix agardhii* (2), *Fischerella sp.* (1), and *Planktothrix rubescens* (1). Based on this finding, the *mcyE* gene is dominantly present among *Microcystis aeruginosa* species.

Table 1. Cyanobacteria strains with *mcyE* toxic gene.

Locus Tag	Species	Chromosome	Gene Type	Protein ID	Product
MAE_38610	*Microcystis aeruginosa* NIES-843	AP009552.1	protein_coding	BAG03683.1	McyE protein
MICAD_2120012	*Microcystis aeruginosa* PCC 7941	HE973164.1	protein_coding	CCI06833.1	Polykeitde synthase and peptide synthetase
MICAH_3180005	*Microcystis aeruginosa* PCC 9809	HE973756.1	protein_coding	CCI24746.1	McyE protein
ANA_C10981	*Anabaena sp.* 90	CP003284.1	protein_coding	AFW93771.1	polyketide synthase peptide synthetase fusion protein McyE
C789_1550	*Microcystis aeruginosa* DIANCHI905	AOCI01000068.1	protein_coding	ELS48657.1	polyketide synthase peptide synthetase fusion protein McyE
A19Y_2227	*Planktothrix agardhii* NIVA-CYA 126/8	CM002803.1	protein_coding	KEI67170.1	McyE
BH695_3240	*Microcystis aeruginosa* PCC 7806SL	CP020771.1	protein_coding	ARI82519.1	McyE
NIES4106_53890	*Fischerella sp.* NIES-4106	AP018299.1	protein_coding	BAZ70594.1	McyE protein
BGM30_08860	*Microcystis aeruginosa* NIES-298	BEYQ01000002.1	protein_coding	GBD51793.1	McyE protein
MSj_00200	*Microcystis aeruginosa* Sj	BDSG01000003.1	protein_coding	GBL08726.1	microcystin synthetase E
myaer102_13370	*Microcystis viridis* NIES-102	AP019314.1	protein_coding	BBH38824.1	McyE protein
PA905_37870	*Planktothrix agardhii* CCAP 1459/11A	BJCD01000059.1	protein_coding	GDZ95487.1	McyE protein
PL11201_770031	*Planktothrix sp.* PCC 11201	LT797711.1	protein_coding	SKB15661.1	NRPS/PKS hybrid enzyme%2C involved in microcystin biosynthesis
PLAN_30459	*Planktothrix rubescens* NIVA-CYA 18	LR812490.1	protein_coding	CAC5343237.1	NRPS/PKS hybrid enzyme%2C involved in microcystin biosynthesis

Source: CyanoOmicsDB (http://www.cyanoomics.cn/) and Zhou *et al.* (2021).

In all strains, the gene type that has been identified is protein-coding. In the same database, there were 4 *Microcystis sp.*, 36 *Microcystic aeruginosa*, 2 *Microcystis viridis*, and 2 *Microcystis flos-aquae* species with homologous *mcyE* genes (>=95% identity) (Table 2).

Table 2. Homologous genes of *Microcystic aeruginosa* NIES-298.

Locus Tag	Identity (%)	Product	Species
D3800_RS11655	100	hybrid non-ribosomal peptide synthetase/type I polyketide synthase	*Microcystis aeruginosa* NIES-298
BGM30_RS04660	100	hybrid non-ribosomal peptide synthetase/type I polyketide synthase	*Microcystis aeruginosa* NIES-298
NIES298_RS07450	100	hybrid non-ribosomal peptide synthetase/type I polyketide synthase	*Microcystis aeruginosa* NIES-298
D3800_11660	100	hybrid non-ribosomal peptide synthetase/type I polyketide synthase	*Microcystis aeruginosa* NIES-298
NIES298_14390	100	microcystin synthetase E	*Microcystis aeruginosa* NIES-298
BKX91_RS02855	99.192	hybrid non-ribosomal peptide synthetase/type I polyketide synthase	*Microcystis aeruginosa* NaRes975
MICAG_RS14440	99.192	hybrid non-ribosomal peptide synthetase/type I polyketide synthase	*Microcystis aeruginosa* PCC 9808
MICAG_2250005	99.192	Microcystin synthetase E	*Microcystis aeruginosa* PCC 9808
MICAD_RS11650	98.962	hybrid non-ribosomal peptide synthetase/type I polyketide synthase	*Microcystis aeruginosa* PCC 7941
MICAD_2120012	98.962	Polykeitde synthase and peptide synthetase	*Microcystis aeruginosa* PCC 7941
IQ224_RS00025	98.356	amino acid adenylation domain-containing protein	*Microcystis sp.* LEGE 00066
IQ224_00025	98.356	amino acid adenylation domain-containing	*Microcystis sp.* LEGE 00066

		protein	
BH695_RS15205	98.327	hybrid non-ribosomal peptide synthetase/type I polyketide synthase	*Microcystis aeruginosa* PCC 7806SL
C789_RS06470	98.327	hybrid non-ribosomal peptide synthetase/type I polyketide synthase	*Microcystis aeruginosa* DIANCHI905
BH695_3240	98.327	McyE	*Microcystis aeruginosa* PCC 7806SL
C789_1550	98.327	polyketide synthase peptide synthetase fusion protein McyE	*Microcystis aeruginosa* DIANCHI905
MICAF_RS11460	98.356	hybrid non-ribosomal peptide synthetase/type I polyketide synthase	*Microcystis aeruginosa* PCC 9807
MICAF_2940006	98.356	Microcystin synthetase E	*Microcystis aeruginosa* PCC 9807
MICAC_RS11630	98.183	hybrid non-ribosomal peptide synthetase/type I polyketide synthase	*Microcystis aeruginosa* PCC 9443
MICAC_3290003	98.183	Microcystin synthetase E	*Microcystis aeruginosa* PCC 9443
IQ242_RS00210	98.212	amino acid adenylation domain-containing protein	*Microcystis sp.* LEGE 08355
IQ242_00210	98.212	amino acid adenylation domain-containing protein	*Microcystis sp.* LEGE 08355
MSj_RS01070	98.067	amino acid adenylation domain-containing protein	*Microcystis aeruginosa* Sj
MSj_00200	98.067	microcystin synthetase E	*Microcystis aeruginosa* Sj
H0902_RS13655	98.096	amino acid adenylation domain-containing protein	*Microcystis aeruginosa* BLCCF108
H0902_13655	98.096	amino acid adenylation domain-containing protein	*Microcystis aeruginosa* BLCCF108
BKX97_RS02405	98.067	hybrid non-ribosomal peptide synthetase/type I polyketide synthase	*Microcystis aeruginosa* CHAOHU 1326
MAE_RS16675	97.894	hybrid non-ribosomal peptide synthetase/type I polyketide synthase	*Microcystis aeruginosa* NIES-843
MAE_38610	97.894	McyE protein	*Microcystis aeruginosa* NIES-843
B1L04_RS28700	97.693	hybrid non-ribosomal peptide synthetase/type I polyketide synthase	*Microcystis aeruginosa* KW
B1L04_29150	97.693	non-ribosomal peptide synthetase	*Microcystis aeruginosa* KW
H6G48_RS20435	97.635	amino acid adenylation domain-containing protein	*Microcystis flos-aquae* FACHB-1344
myaer102_RS07280	97.721	amino acid adenylation domain-containing protein	*Microcystis viridis* NIES-102
H6G48_20430	97.635	amino acid adenylation domain-containing protein	*Microcystis flos-aquae* FACHB-1344
myaer102_13370	97.721	McyE protein	*Microcystis viridis* NIES-102
MAE30S32_RS00060	97.635	amino acid adenylation domain-containing protein	*Microcystis aeruginosa* 11-30S32
MAE30S32_00100	97.635	hybrid non-ribosomal peptide synthetase/type I polyketide synthase	*Microcystis aeruginosa* 11-30S32
MICAH_RS11990	97.664	hybrid non-ribosomal peptide synthetase/type I polyketide synthase	*Microcystis aeruginosa* PCC 9809
MICAH_3180005	97.664	McyE protein	*Microcystis aeruginosa* PCC 9809
OA58_RS11865	97.404	hybrid non-ribosomal peptide synthetase/type I polyketide synthase	*Microcystis aeruginosa* NIES-88
OA58_11945	97.404	thioester reductase	*Microcystis aeruginosa* NIES-88
MiTe_RS02520	97.404	amino acid adenylation domain-containing protein	*Microcystis aeruginosa* NIES-2520
MiTe_00465	97.404	tyrocidine synthase 3	*Microcystis aeruginosa* NIES-2520
MAESPC_05048	98.973	Beta-ketoacyl-acyl-carrier-protein synthase I	*Microcystis aeruginosa* SPC777

Source: CyanoOmicsDB (http://www.cyanoomics.cn/), Zhou *et al.* (2021).

However, there were nine (9) different gene products described in the database (Fig. 4). These gene products include hybrid non-ribosomal peptide synthetase/type I polyketide synthase, microcystin synthetase E, polyketide synthase and peptide synthetase, amino acid adenylation domain-containing protein, polyketide synthase peptide synthetase fusion protein McyE, non-ribosomal peptide synthetase, thioester reductase, tyrocidine synthase 3, and beta-ketoacyl-acyl-carrier-protein

synthase I. The most frequently expressed protein by the *mcyE* gene is the hybrid non-ribosomal peptide synthetase/type I polyketide synthase protein product (39%). Furthermore, the second most frequently expressed is the microcystin synthetase E (21%) while the rest of the protein products are rarely expressed.

The production of microcystin has been found by Dittmann *et al.* (2013) to be distantly related to cyanobacterial genera which can be explained by the *mcyE* gene cluster relative to its evolutionary history and diversification. In its phylogenetic analysis, the *mcy* gene clusters must have evolved from a common ancestor according to Rantala *et al.* (2004). It was further validated by Tooming-Klunderud *et al.* (2008) that this gene cluster has not only diversified during the process of speciation but by numerous recent intragenomic recombination events and point mutations. Moreover, microcystin-leucine arginine (MC-LR) is a very common and potent variant among cyanotoxins. Several pieces of research have found that a more concentrated MC-LR in a certain medium in the environment can induce oxidative stress which can initiate toxicity in the liver, gonadal, and nervous system (Hu *et al.*, 2016; Valerio *et al.*, 2016).

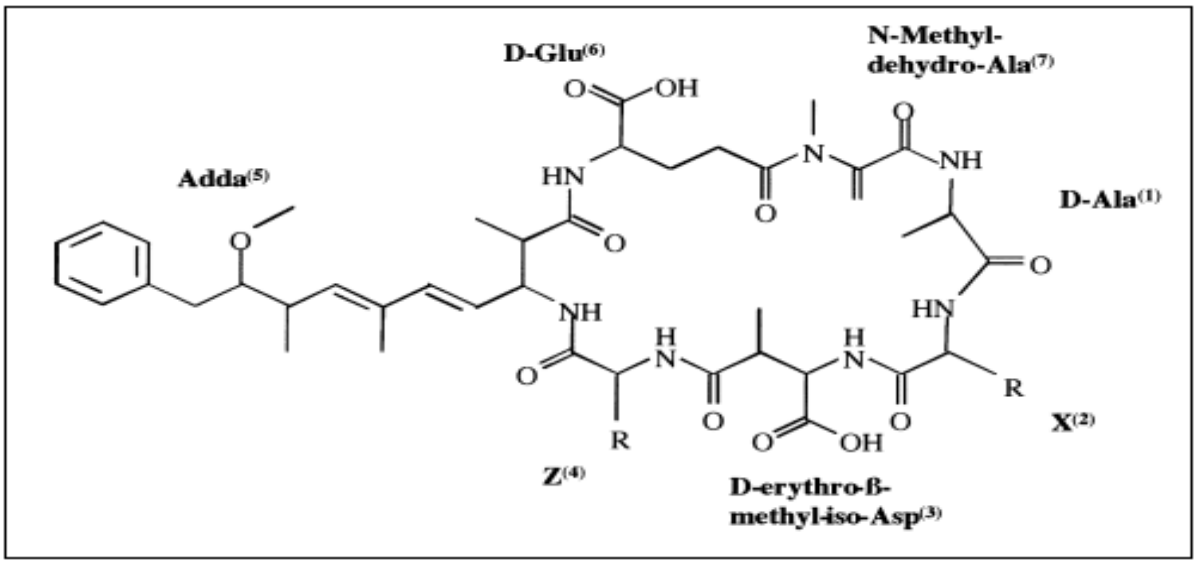

Fig. 1. The general structure of the microcystin. *Source*: Tillet *et al.*, 2000.

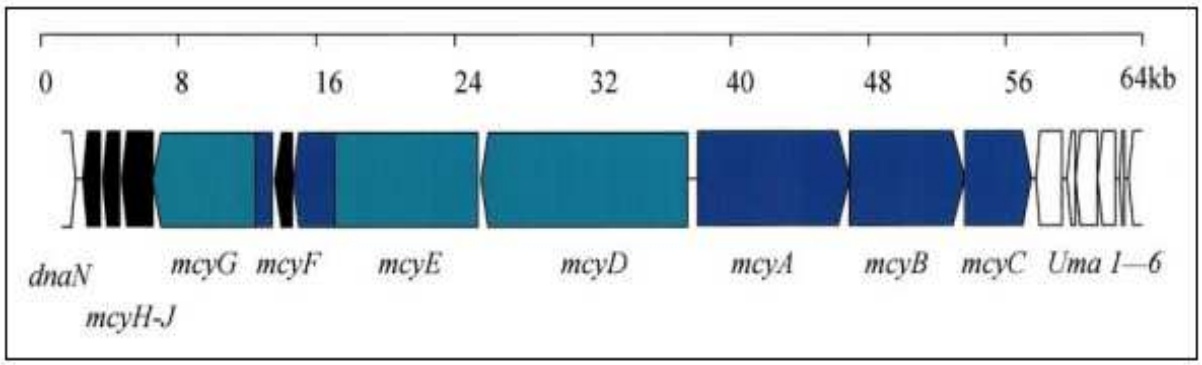

Fig. 2. Gene cluster organization in the biosynthesis of microcystin.

In a recent study conducted by Liu *et al.* (2018), the toxicity of MC-LR can induce cells apoptosis and morphology changes mainly in reproduction, oxidative stress when the generation of reactive oxygen species (ROS) is stimulated, destroy antioxidant capacity, and trigger endoplasmic reticulum stress (ERs) and autophagy by overexpressing ATG12, ATG5, ATG16, EIF2α (phosphorylated at S51), CHOP, XBP1, GRP78, Beclin1, and PERK (Thr980). Based on the physical

and chemical structures of the microcystin-LR, all these effects can be carried out by the molecular interactions essential for cellular activities. The *mcyE* toxic gene is common in *Microcystis aeruginosa* (7), *Microcystis viridis* (1), *Anabaena sp.* (1), *Planktothrix sp.* (1), *Planktothrix agardhii* (2), *Fischerella sp.* (1), and *Planktothrix rubescens* (1). The protein-coding genes and their exact number among these species may vary because of different genetic processes which can produce different variants of RNA. According to Harrow *et al.* (2009), the protein-coding genes made most of the RNA sequences after translation and its complete set is important to be known.

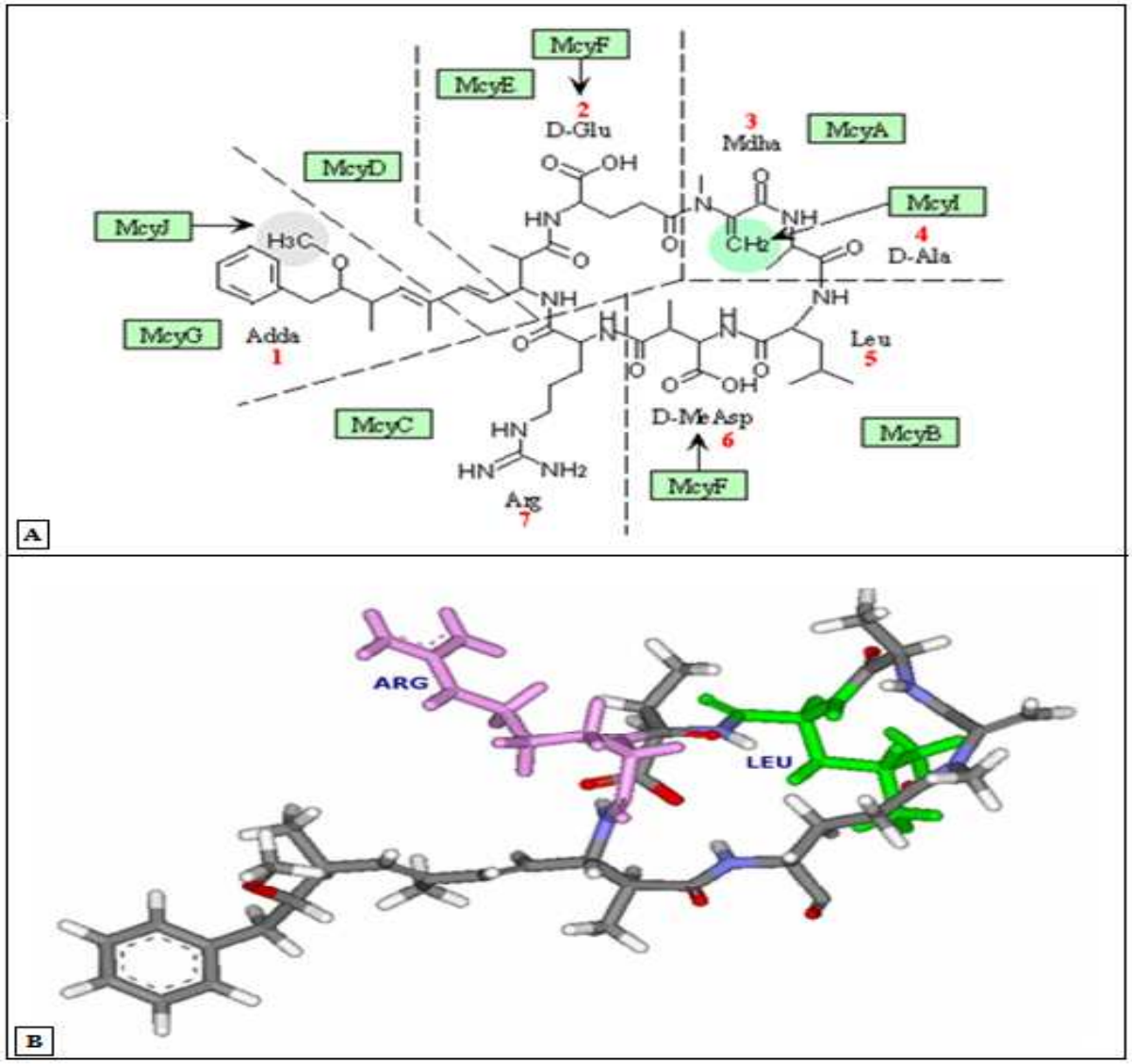

Fig. 3. Structure of the cyanobacterial toxin (microcystin-LR) showing a non-ribosomal peptide structure (A) and chemical structure of oligopeptidic toxin MCYST-LR where two amino acid residue ARG and LEU is shown in magenta and green color, respectively (B). *Source*: KEGG, Kanehisa *et al.*, 2000; Singh *et al.*, 2016.

Based on the database, the gene products include hybrid non-ribosomal peptide synthetase/type I polyketide synthase, microcystin synthetase E, polyketide synthase and peptide synthetase, amino acid adenylation domain-containing protein, polyketide synthase peptide synthetase fusion protein McyE, non-ribosomal peptide synthetase, thioester reductase, tyrocidine synthase 3, and beta-ketoacyl-acyl-carrier-protein synthase I. Among these gene products, the most abundant is the hybrid non-ribosomal peptide synthetase/type I polyketide synthase and microcystin synthetase E.

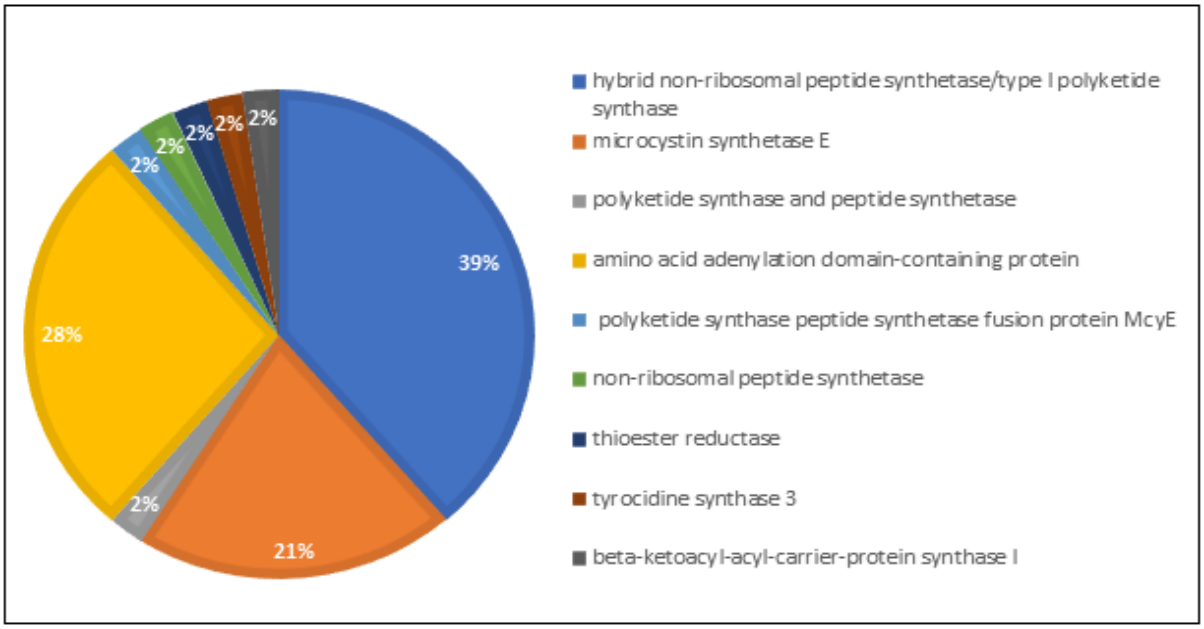

Fig. 4. Summary of protein products produced by the *mcyE* gene of *Microcystic aeruginosa* NIES-298.

The polyketide synthases and non-ribosomal peptide synthetases are multi-functional enzymes related to the modular organization to collaborate and assemble complex polyketides or non-ribosomal polypeptides in linear form (Hwang *et al.*, 2020). Compounds produced from reductase-terminated gene clusters are seen as reactive and function mainly in biological activities. Thioester reductase produces an aldehyde or alcohol to form macrocyclic imine and acts as an intermediary in the biosynthesis of polyketide alkaloids and pyrrolobenzodiazepines (Mullowney *et al.*, 2018). Still, there is little information about the mechanisms and roles of these different protein products in the production of microcystin toxins. When these compounds become active, they exhibit biological activities that are relevant to the natural environment like antifungal, antiviral, antibacterial, and cytotoxic activities (Kaushik *et al.*, 2009; Portmann *et al.*, 2009).

Conclusion

Microcystin is a heptapeptide wherein its toxin structure consists of a non-ribosomal peptide structure with arginine and leucine. There are fourteen (14) species that have been found with the *mcyE* toxic gene. These species include strains that are identified as *Microcystis aeruginosa* (7), *Microcystis viridis* (1), *Anabaena sp.* (1), *Planktothrix sp.* (1), *Planktothrix agardhii* (2), *Fischerella sp.* (1), and *Planktothrix rubescens* (1). The *mcyE* gene is dominantly present among *Microcystis aeruginosa* species. There were nine (9) gene products found in the database of CyanoOmicsDB. These gene products include hybrid non-ribosomal peptide synthetase/type I polyketide synthase, microcystin synthetase E, polyketide synthase and peptide synthetase, amino acid adenylation domain-containing protein, polyketide synthase peptide synthetase fusion protein McyE, non-ribosomal peptide synthetase, thioester reductase, tyrocidine synthase 3, and beta-ketoacyl-acyl-carrier-protein synthase I.

These proteins are active in different biological activities, specifically, in the organization and collaboration of complex polyketides or non-ribosomal polypeptides. However, there is a need to associate these different protein products with the production of microcystin toxins among the cyanobacteria. Understanding the mechanisms of these different protein products may contribute to the solution of regulating the release of microcystins in the environment. This, in turn, will protect human health as well as the environment exposed to different pollution sources that can stimulate the production of microcystin toxins.

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