Mass-producing microcystin-LR-degrading enzymes for algal bloom toxic remediation

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Algal blooms are the excessive growth of algae that causes low oxygen levels in the water. They are detrimental to aquatic ecosystems and organisms that consume the water. Cyanobacteria are a type of bacteria that can photosynthesize, but many also produce toxins in their surrounding environment. Harmful algal blooms containing the cyanobacteria *Microcystis aeruginosa*, like those in Lake Erie, produce the toxin microcystin-LR. This toxin disrupts cellular pathways by interfering with serine and threonine phosphatases in cells. Phosphatases are vital enzymes that remove phosphates from many proteins involved in various biochemical pathways. The inhibition of phosphatases by toxins is a leading cause of liver and kidney damage, which can result in death. This toxin's other adverse effects include increased tumorigenicity in humans, fish, and birds. Consumption can also lead to pneumonia, vomiting, abdominal pain, oral blisters, dry coughing, diarrhea, headaches, and sore throats. Microcystin poisoning currently has no cure. In 2014, a Lake Erie toxic algal bloom prompted health officials to advise the Toledo community to avoid contact with tap water due to a high concentration of microcystins in the water system. Our goal is to modify *Escherichia coli* to mass-produce five enzymes, MlrA, MlrB, MlrC, MlrE, and MlrF, to remediate the toxin. These enzymes, found in species of *Sphingopyxis*, can break down the toxin into innocuous pieces. MlrA linearizes the ring structure of toxic microcystin-LR, while MlrB breaks the linear microcystin-LR into a tetrapeptide, and MlrC degrades the tetrapeptides into smaller peptides and amino acids. MlrE and MlrF are not well characterized but are needed to degrade the toxin. Our project would produce large quantities of the five enzymes to break down the toxin. The application of these enzymes would be a spray directly onto algal blooms or a filter impregnated with the enzymes in water treatment facilities.

Keywords: microcystin-LR, algal blooms, Lake Erie, remediation

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Watch a video introduction by the authors at https://youtu.be/NZT-Zx8yALE

Background

Harmful algal blooms (HABs), which have been recorded for centuries, affect many ecosystems (hab.whoi.edu, 2022). Factors such as significant nutrient pollution and agricultural run-off augment the concentration of phosphorus and nitrogen in water sources, stimulating algal bloom (*gov.bc.ca, 2022*). Other driving factors of these blooms include warmer temperatures, abundant light, and stable wind conditions (*gov.bc.ca, 2022*).

Lake Erie provides the perfect environment for algal growth due to its warm, shallow waters and surrounding agricultural lands. Agricultural practices around this body of water use large amounts of fertilizer that run-off into the lake at a higher rate than any other lakes (glslcities.org, 2022). These factors greatly contributes to the severity of Lake Erie's algal blooms.

The harmful effects of the algal blooms catalyze fish migration. According to the Integrated Risk Assessment Branch's study, the blooms consume oxygen, block sunlight, and produce detrimental toxins production which establish dangerous habitats. Moreover, the aforementioned effects significantly impact human health and activity in Lake Erie's surrounding communities (Ioehha.ca.gov, 2009).

One of the most prevalent examples of this recurring threat can be observed in Toledo, Ohio. The Toledo community sources its water directly come from Lake Erie and the Maumee River. Water treatment facilities cannot purify the toxins created by algal blooms, which poison those who rely on the lake's water supply (*Rogener*, 2020). In 2014, a combination of heavy winds and an HAB prompted citywide orders to pause the use of all forms of tap water for three days. The harsh winds caused waves of water to directly flow into water treatment facilities, raising the microcystin level to over 1.0 ppb: a quantity the World Health Organization deems toxic for humans. This halt in access to water was a significant burden on the eleven million people who depend on Lake Erie water (glslcities.org, 2022).

Microcystin-LR (MC-LR) is the most harmful toxin produced by Cyanobacteria. Consuming animals or fish contaminated with MC-LR, swimming in an area containing HABs, and boating in contaminated water may result in MC-LR exposure (McAbee *et al.*, 2017). *Microcystis aeruginosa* releases the MC-LR toxin when its cell walls burst during death. MC-LR is a cyclic heptapeptide made up of seven amino acids: D-alanine (D-Ala), L-amino acid (L-Leu), D-Methylaspartic acid (D-Me Asp), L-amino acid (L-Arg), β amino acid (Adda), D-Glutamic acid (D-Iso Glu), and N-Methyl Dehydroalanine (Moha) (Schneegurt, 2022) In normal bodies of water, this stable structure allows MC-LR to be resistant to common chemical breakdowns such as hydrolysis or oxidation. Though MC-LR is vulnerable to some bacterial proteases, such bacteria are not naturally prevalent in Lake Erie. In addition, boiling is insufficient to filter the water as microcystins can withstand extreme temperatures (oehha.ca.gov, 2009).

Greer *et al.*'s recent study (2018) demonstrated the absorption levels of MC-LR. They exposed a pig to MC-LR to mimic and identify the toxin's effect on the human body. Two groups were involved in the experiment for comparison. One of the experimental groups took eight samples of 0.04 μg/kg bw MC-LR daily for 13 weeks, while scientists administered doses of 2 μg/kg bw MC-LR daily to the second group of the same species for five weeks. All other variables remained unchanged. At the end of the experiment, 50% of the samples from the higher-dosed group indicated accumulation and absorption of MC-LR in the liver tissue, averaging 1.1% of the intake dose, which resulted in significant liver damage (Greer *et al.*, 2018).

Microcystins denature enzymes known as protein phosphatases. These enzymes perform common steps in many biological pathways, such as removing phosphates from amino acids like serine and threonine. When MC-LR inhibits these enzymes' function, the toxin causes a build-up of phosphorylated protein. This can induce cell apoptosis, leading to liver cell death. This mechanism is likely how MC-LR destroys livers. Evidence indicates that MC-LR increases other proteins in pathways leading to apoptosis (Yoshida *et al.*, 1997).

MlrA, MlrB, MlrC, MlrD, MlrE, and MlrF are a set of enzymes that degrade MC-LR. These enzymes are naturally found in *Sphingomonas* bacteria, which reside primarily in soil and water. The bacteria degrade microcystin to obtain a carbon source (Savola, 2016). Past experiments observed the successful heterologous expression of MlrA in *E. coli* (Lee *et al.*, 2006). MlrA linearizes the cyclic nature of MC-LR through hydrolysis. During this process, the peptide bond between β amino acid (ADDA) and L-arginine (L-Arg) breaks apart to form a linearized product (Savola, 2016). Following this, MlrB, a serine protease, breaks apart the bond between the Leucine (L-Leu) and D-alanine amino acids to form a tetrapeptide (Massey & Yang, 2020). MlrC, a metalloprotease, further degrades the tetrapeptide into amino acids (Savola, 2016). MlrD aids in the transport of microcystin-LR into the cell (Massey & Yang, 2020). The specific functions of MlrE and MlrF are unknown, but they are critical to microcystin-LR degradation (Metzloff, 2019a; 2019b).

Our project will insert genes for the enzymes MlrA, B, C, E, and F into five different DH5α *E. coli* optimized

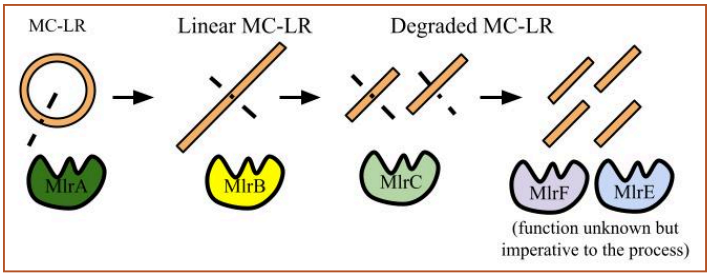

Figure 1: *The breakdown of microcystin-LR through MlrA, B, C, E, and F enzymes.*

for plasmid production (mclab, 2022). The plasmid will then be isolated from DH5α using plasmid prep and transformed into a second strain of *E. coli,* T7 Express Competent *E.coli,* for maximum efficiency protein production (NEB, 2022a). We will not use MlrD because this enzyme's function is to import the toxin into a cell. Instead, the transformation will include the enzymes produced within the bacteria to break down the microcystin toxin. This project will use one plasmid backbone, pET-28a(+), which contains a T7 promoter, a ribosomal binding site, a T7 terminator, a gene coding for kanamycin resistance, an N-terminal his-tag, and a thrombin tag. We will clone each mlr gene into its respective pET-28a(+) backbone to construct five unique plasmids (Sigma-Aldrich, 2022). Each unique plasmid will be transformed into DH5α, creating five different plasmids (*Figure 1*). The project aims to mass-produce and purify the enzymes, allowing us to test them for optimal enzymatic activity against the toxin. We envision utilizing these enzymes to directly remediate toxin-producing algal blooms and bolster filtration mechanisms in water treatment facilities.

Systems Level

This design introduces the genes encoding the microcystin-degrading enzymes MlrA, MlrB, MlrC, MlrE, and MlrF into DH5α *E. coli*. We will clone the individual gene for each enzyme into five separate copies of the pET-28a(+) plasmid, which contains the T7 promoter, the T7 terminator, kanamycin resistance, an N-terminal his-tag, and a thrombin tag (Sigma-Aldrich). Each of these plasmids will be transformed into separate electrocompetent DH5α *E. coli* via electroporation. Using kanamycin as a selectable marker, we will culture our transgenic *E. coli* to ensure the presence of the plasmid. The DH5α will then be lysed with chemical detergent to extract the produced plasmids using plasmid prep which will then be transformed into the T7 Express Competent *E. coli* strain optimized for protein production (NEB, 2022a).

Upon successful transformation, *E. coli* will become a factory for enzyme production. A complimentary detergent solution to the IMAC column will lyse the *E. coli*. To purify the desired Mlr enzymes, the transformed, lysed *E. coli* will be poured over a nickel IMAC column to undergo low-pressure chromatography (*Figure 2*). The his-tagged enzymes will attach to the immobilized ions on the side of the IMAC column. We will dispose of the unwanted residue and add an eluting agent within the column to release the his-tagged enzymes (ThermoFisher Scientific). This process will result in five test tubes, each containing a purified MlrA, B, C, E, or F enzyme. The enzymes will be tested in a controlled lab environment containing the microcystin-LR toxin to determine the optimal enzyme expression levels.

Device Level

The DH5α E.coli strain will be used to mass produce 5 distinct plasmids. These 5 plasmids will then be transformed into the T7 Express *E.coli* strain, which will act as the chassis to aid in the mass production of MlrA, MlrB, MlrC, MlrE, and MlrF. The enzyme MlrD is unnecessary in the design, as its primary function is to import the toxin (*Part:BBa_K1520000*, 2014). Although the specific functions of MlrE and MlrF are unknown, they are seemingly crucial to the breakdown of MC-LR (*Part:BBa_K2960010*, 2019; *Part:BBa_K2960011*, 2019). To meet the goal of using the enzymes to treat the toxin in situ, we must produce the enzymes safely and efficiently. The T7 Express strain of *E. coli* is a well-tested chassis, making it a suitable host for enzyme production, while the DH5α strain is optimal for the cloning of plasmids. (Kostylev *et al.*, 2015; NEB, 2022a). The project utilizes the plasmid backbone pET-28a(+), which contains a T7 promoter, an N-terminal his-tag, a thrombin tag, a ribosomal binding site, BamH1 and EcoR1 restriction enzymes, and a T7 terminator (Figure 3.). This plasmid has a selectable marker of bacterial resistance for kanamycin (*Plasmid: pET-28*).

Parts Level

Our goal is to produce the enzymes MlrA, B, C, E, and F by inserting their respective genes into plasmid pET-28a(+), an ideal system (Figure 4.) for the cloning and expression of recombinant proteins in *E. coli* (Sigma-Aldrich). The multi-cloning site for this plasmid contains abundant restriction sites, offering diverse opportunities for the restriction enzyme choice. In addition, the Kan coding sequence on the plasmid provides resistance to the kanamycin antibiotic, which will act as the selectable marker used to identify transformed *E. coli*. The promoter of the plasmid, T7, is recognized by the T7 RNA polymerase and allows high expression levels of the genes. A his-tag will be added to the N-terminal

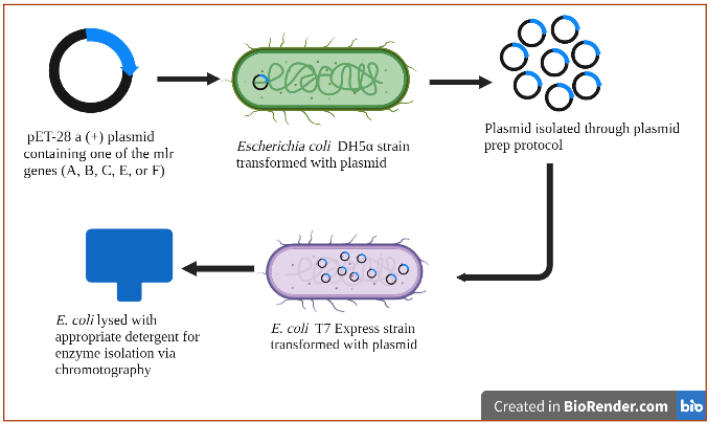

Figure 2. *The five pET-28a(+) plasmids, each containing one of the mlrA, B, C, E or F genes, are inserted into an E. coli to promote the mass production of the Mlr enzymes.*

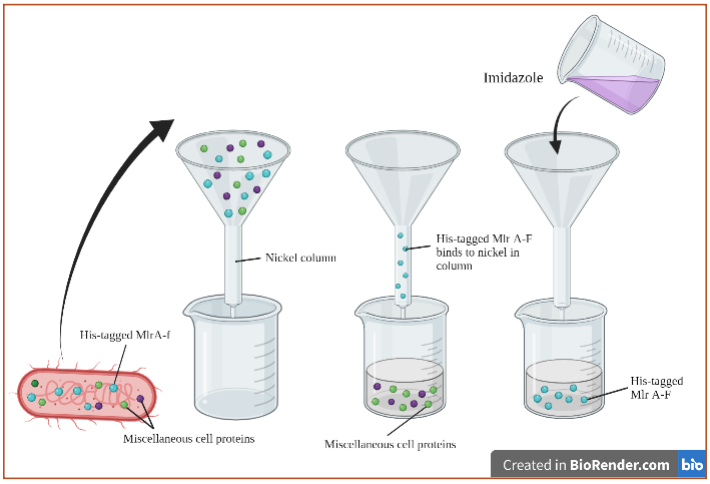

Figure 3. *Low-pressure liquid chromatography willisolate the histidine-tagged MlrA, MlrB, MlrC, MLrE, and MlrF enzymes from the DH5α E. coli strain containing the plasmid. Transformed E. coli from the overnight culture are lysed and the lysate (miscellaneous cell proteins, including histidine-tagged Mlr enzymes), will be filtered through a nickel column. His-tagged Mlr enzymes will bind to the nickel while other cell proteins pass through. The Mlr enzymes will then be eluded with a high concentration of Imidazole to purify the enzymes.*

of the gene to enable the purification of enzymes. A thrombin tag will be useful and potentially necessary if our purified enzymes are not functioning with the his-tag still attached. Of the multiple restriction sites in the MCS, we will use BamHI and EcoRI to insert the genes for our enzymes. Any internal EcoRI and BamHI restriction sites will be altered during synthesis to avoid cleavage of each gene during cloning. We will insert the gene of interest using these two restriction sites. Oncethe mlrA, B, C, E, and F genes are inserted into the plasmid, *E. coli* will produce the desired MlrA, B, C, E, and F enzymes.

Safety

E. coli has been a primary model organism in biological research for decades). The DH5α strain of *E.coli* has a high transformation efficiency and a mutation that increases the stability of genetic inserts. In addition, DH5α is one of the most commonly used strains for genetic modification (mclab, 2022. Classified as a Biosafety Level 1 strain, DH5α should have no harmful effects on healthy humans.

The T7 Express strain of *E.coli* is commonly used for the over-expression of proteins and has a high transformation efficiency. It is classified as a Biosafety Level 1 organism, so it should present minimal risk to healthy humans (NEB, 2022b).

Additionally, this project requires the usage of microcystin-LR. Microcystins are potent liver toxins and possible human carcinogens (Lone *et al.*, 2015). Specifically, microcystin-LR is highly toxic in cases of inhalation and skin and eye contact (EPA, 2010). We will conduct all procedures utilizing the microcystin-LR in an adequately ventilated lab, alongside the use of full personal protective equipment (Abmole Bioscience Inc). After the experiment, the toxin will be disposed by following the US Environmental Protection Agency's guidelines for the disposal of environmental laboratory samples.

Discussions

In a lab, we will test concentrations for each enzyme on microcystin-LR samples to create the optimal ratio for treatment. The purified enzymes will be tested for their efficacy in breaking down MC-LR into harmless amino acids in a laboratory setting with cyanobacteria. Once we have obtained the desired effects of the enzymes, we will further test on small ponds suffering from microcystin algal blooms before introducing the enzymes into Lake Erie. If our project is successful, it can protect people worldwide by creating a cleaner and healthier water source.

In order to create this safe water source, we also need to consider the potential hazards during practical use. Research has verified enzymes MlrA, B, C, E, and F for the decomposition of the MC-LR, but the excessive amount of enzymes we plan to introduce may adversely affect other organisms in Lake Erie. Before introducing any practical applications using the enzymes, consultation with experts and safety experiments to confirm the enzyme's effects on other organisms would be necessary. Initially, this project considered

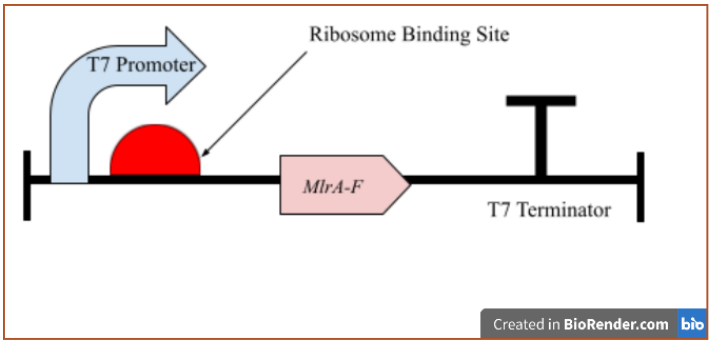

Figure 4. *This genetic construct will be repeated for each mlr genes used for production.*

alternatives to spreading Mlr enzymes, such as modifying a native organism in Lake Erie or editing a cyanophage to produce these enzymes.

Our team also found an operon containing the mlrA, B, and C genes (Mikalson *et al.*, 2003). With a plasmid, a system using one *E. coli* for the mlrA, B, C operon would be possible, but this approach makes it challenging to produce various concentrations of these enzymes. Using five separate *E. coli*, we can optimize the concentrations of the enzymes.

One possible application of this project would be transforming *Pseudomonas lutea*, a native Lake Erie organism, with the modified plasmid. *P. lutea* has been shown to contain the mlrA, mlrB, and mlrC genes. We would modify the organism to contain the mlrE and mlrF genes, while also upregulating the expression of its preexisting mlr enzymes. Over time, we can extend the application of these designs into broader fields. We can widen this project's scope to other regions and bodies of water. Moreover, this design would open a pathway to discovering new mechanisms for the degradation of toxins other than microcystins. Recent studies suggest that nodularin is a toxin that exhibits a similar structure to microcystin and has detrimental effects on numerous organisms (Pearson *et al.*, 2010). The same approach of microcystin elimination can be applied to other toxins and can potentially improve other aspects of the ecosystem.

Next Steps

Currently, our design remains on a theoretical level. The first step of testing the effectiveness of the five enzymes is to use a mix of the bacterial lysate of the five strains directly on commercially available Microcystin-LR (*Microcystin-LR*). To further research its practical applications, we will test MlrA, B, C, E, and F'sfunctions with a his-tag. Previous studies have shown that the presence of a his-tag on the MlrA, B, and C enzymes

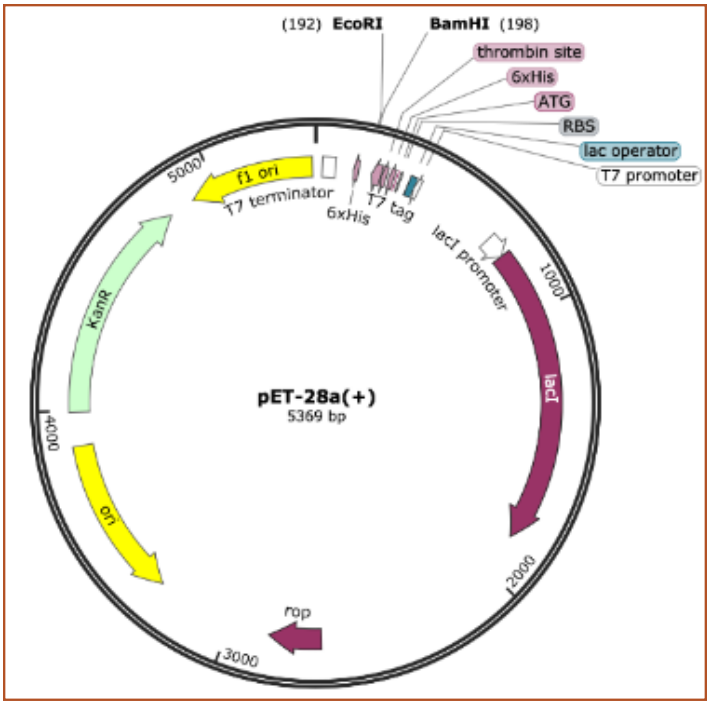

Figure 5. *The genes mlrA, B, C, E and F will be inserted into separate pET-28 plasmids between EcoRI and BamHI. This plasmid is an ideal component of our experimental design as it is suitable for the mass-production of the enzymes.*

will not impact their respective functions (Fionah *et al.*, 2022; Nybom *et al.*, 2016). Therefore, removing the his-tag from MlrA, B, and C is unnecessary. The specific characteristics and functions of MlrE and MlrF are still unclear to researchers today. However, mlrE and mlrF are found in the same genome of the *Sphingopyxis* sp. strain C-1 as mlrA, B, and C (Okano *et al.*, 2020). We hypothesize that removing the his-tag from the MlrE and MlrF enzymes is unnecessary. Therefore, we can use complete enzymes in further experiments. In addition, it is necessary to test the proportion of each enzyme in relation to one another to determine the optimal enzyme concentrations for toxin breakdown. If our design proves to be experimentally successful, we hope to further our efforts to remediate the effects of the microcystin toxin in Lake Erie.

Author Contributions

R.R. came up with the original idea and began introductory research. L. A., A. B., E. B., E. F., S. M., N. H., K. L., A. O., and Y. Z. conducted the early research process. L.A., A. B., E. B., B. C., E. F., S. M., I. H., D. L., J. L., Y. L., K. L., A. N., C. X., S. Y., M. Z., and Y. Z. contributed to the writing and proofreading of the paper. A. B., E. F., I. H., D. L., J. L., Y. L., C. X., and M. Z. designed the images and graphics for this project and worked on the video.

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