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Enzymatic Degradation of Microcystin-LR by Microcystinase (MlrA)

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Enzymatic Degradation of Microcystin-LR by Microcystinase (MlrA)

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by
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Abstract

Microcystin-LR (MC-LR) is affecting the water supply worldwide. Hence, a way to eliminate this toxin is an essential target. In this study, successful cloning of the *mlrA* gene and producing MlrA enzyme that can degrade the cyclic MC-LR to linearized MC-LR was done. MlrA protein was expressed in *Escherichia coli* BL-21 (*E. coli*). Also, enhancing the MlrA yield by adding nickel to LB media was a success in producing more MlrA enzyme from the same volume. Even though the enzyme showed no activity after adding Ni, the enzyme was expressed at a higher yield. Furthermore, it was to investigate adding methanol to the Sulfonated poly (ether ether ketone) (SPEEK) membrane, which was used for the immobilization of MlrA, to desorb the MC-LR from the membrane when doing the experiment of the immobilized MlrA. Lastly, investigating the enzyme activity before and after enhancing the protein yield and showing that adding the nickel will hinder the MlrA activity while the enzyme that produced in the same conditions without Ni was active.

Acknowledgments

I want to thank my merciful God for all his blessing and the help I got to finish this thesis. Foremost, I want to express my special thanks to my advisor, Dr. Audie Thompson, for all the support, guidance, and encouragement to finish my projects. Additionally, I want to thank her for making me part of Dr. Thompson's research group. Another special thank is to Dr. Hazim Aljewari for all his effort and his great endless assistance. Also, I want to thank my colleagues, Raquel De Castro, and Cannon Hackett, for their help every time I ask.

This thesis is dedicated to my mother, brothers, and sisters who helped me reach this point in my life. Also, I dedicate this thesis to my wife Eidah and my kids for all their sacrifice and effort to help me focus on my goals. Lastly, this thesis is dedicated to all of my extended family in Saudi Arabia.

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1. Chapter one:

1.1 Introduction on MlrA:

Enzymes are proteins that help accelerate the chemical reactions in living organisms (biocatalysts) by influencing the reaction speed by a million or more times (Berg, Tymoczko, & Stryer, 2012). Microcystinase (MlrA) breaks down microcystin, a cyanobacterial toxin. MlrA is an example of metalloenzymes, which uses a zinc ion as a cofactor in its active site (David G. Bourne, Riddles, Jones, Smith, & Blakeley, 2001; Dexter, Mccormick, Fu, & Dziga, 2021).

MlrA is the first and the most crucial biodegradation step in the microcystin enzymatic degradation pathway. Without MlrA, the cyclic microcystin-leucine arginine (MC-LR) will not be linearized to continue the enzymatic degradation process. MlrA was first described in 1996 (D. G. Bourne et al., 1996), and its activity was proven to be specific to target the peptide bond between the Adda group and the second amino acid in the MCs toxin (Dexter et al., 2021). Until now, MlrA, MlrB, MlrC, and MlrD are the only enzymatic degradation pathway known to eliminate MCs. There is currently an increased interest in this pathway from scientists to find a novel alternative to industrial MCs removal from the water supply. MCs are cyanobacteria products that provide an environment free from opposition bacterium and help cyanobacteria in photosynthesis. In nature, MCs need 2 to 8 days for the degradation to start by other bacteria. From this finding, researchers were able to identify bacteria that have the ability to neutralize the cyanobacteria toxin. Also, researchers found the gene clusters that express the enzymes required for the microcystin enzymatic degradation pathway. From the gene that was isolated from these bacteria, the heterologous of the *mlr* gene in an expression vector was done to increase the expression of the enzymes.

1.2 Enzyme immobilization:

Enzyme immobilization is a technique where enzyme molecules are trapped in solid support as a reactant, the substrate can pass through the enzyme, and the reaction will occur, later the products can be collected (Sirisha, Jain, & Jain, 2016). It is widely used in the industry for a few reasons like the product gatherer does not mix with the enzyme, so an additional technique to separate the enzyme and the products are unnecessary (Homaei, Sariri, Vianello, & Stevanato, 2013). Enzyme molecules recover more rapidly when they are immobilized, so they are ready for the following reaction. In a short time, immobilization also stabilizes the enzyme. So, they are less likely to denature. There are several ways in which enzyme immobilization can be done. These include adsorption, ionic bonding, covalent bonding, and entrapment method (Homaei et al., 2013). An example of an enzyme immobilization vehicle is sulfonated poly (ether ether ketone) (SPEEK) membranes. SPEEK membranes have an average hydrophilic property and stay negatively charged in both acidic and basic conditions (Eke, Elder, & Escobar, 2018).

1.3 Bacteria stress:

E. coli can be stressed by its environment. This stress will force the bacteria to adapt to this new condition or die (Washington – Hughes, Ford, Jones, Mcrae, & Outten, 2019). This stress was caused by the low-intracellular iron levels in *E. coli*. Consequently, *E. coli* will maintain a high expression of its iron uptake systems. Adding Ni to the LB media will cause this stress, making the lag phase longer (Washington – Hughes et al., 2019). By using this property, the MlrA enzyme could be expressed in a high quantity. This stress can be done by adding nickel to LB media to enhance and increase the expression of *mlrA* gen in the host. Free nickel is toxic to

E. coli (Kumar, Mishra, Kaur, & Dutta, 2017; Macomber & Hausinger, 2011; Washington – Hughes et al., 2019); therefore, the right amount of nickel and time has to be determined to stress the host. Since Ni is toxic to *E. coli*, the *E. coli* will start its response to the stress by expressing its essential enzymes to prevent the death of the bacteria. Because Ni damages the DNA, *E. coli* will try to start the SOS response, but Ni will prevent the SOS response in the bacteria (Kumar et al., 2017), which means that bacteria resources may become available to the heterologous enzyme of interest in this case, MlrA. By adding 1.4 mM Ni to the growth culture after 30 min from adding the IPTG, *E. coli* is at its highest activity in the logarithmic phase, which can, in theory, redirect the cell resources to the expression of MlrA. The choose of the Log phase was passed on the previous study on *E. coli* stress (Chung, Bang, & Drake, 2006). Nickel stress will force *E. coli* to increase its metal concentration instead of having the minimum limit for its enzymes (Macomber & Hausinger, 2011; Washington – Hughes et al., 2019). This accumulation of vital metals to *E.coli* would be done during the lag phase if the Ni was added during this phase (Rolfe Matthew et al., 2012).

MlrA is a zinc metalloprotein, and zinc is one of the metals that can be stored at a higher level inside the bacteria during stress. The second possibility is that nickel may substitute zinc in MlrA or bind at the MlrA on another site; it may also inhibit MlrA activity (Macomber & Hausinger, 2011). So, increasing the zinc concentration and overexpressing the host enzymes can lead to high MlrA concentration in the enzyme expression process.

2. Chapter 2: Literature Review:

Microcystin (MC) is a harmful natural bacteria product, and it is contaminating water supplies worldwide. For example, the city of Toledo, Ohio, shut down its entire drinking water system in 2014 because of a cyanobacterial bloom (Steffen et al., 2017). MC contamination is occurring at a fast rate due to global warming and an environment rich in nutrients like nitrogen and phosphorus (Liu et al., 2020; Martínez-Ruiz & Martínez-Jerónimo, 2016). Nitrogen and phosphorus are nutrients that are natural parts of aquatic ecosystems. Nitrogen and phosphorus support the growth of algae and aquatic plants, which provide food and habitat for organisms that live in water.

Furthermore, phosphorus and nitrogen are in an overflowing supply because of pesticides and fertilizers in agriculture. In addition, phosphorus and nitrogen come from water treatment plants. For these reasons, freshwater cyanobacteria producing MC started to colonize environments where the bacterium did not exist (Wilken, Wiezer, Huisman, & Van Donk, 2010).

Consuming water that is contaminated with MC can cause cancer or even death. Many studies were done to study and eliminate MC toxicity from the environment. There are around 250 types of MC, but MC-leucine-arginine (MC-LR) is the most toxic type of MC and is widely available in water sources around the world (Dexter et al., 2021; Yang et al., 2020). MCs are a group of cyclic heptapeptides that contains D-alanine, D-erythro-b-methylaspartic acid, Adda, c-linked-D-glutamic acid, N-methyl dehydroalanine, and two-variable L-amino acids (Chang et al., 2015; Dexter et al., 2021).

There are many ways to degrade MC-LR that are used today. There is a physical treatment, chemical treatment, and biological treatment. All these treatments cannot degrade MC toxicity completely or have side effects (ref).

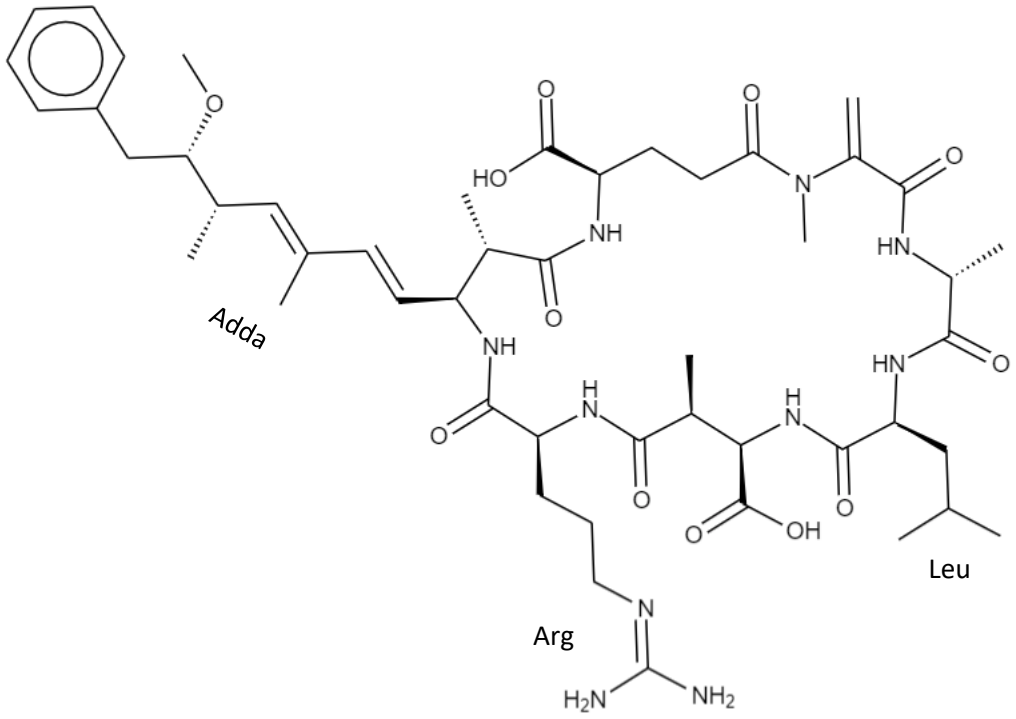

Figure.1: MC-LR cyclic structure

Physical treatment can remove cyanobacteria from water, but it cannot remove MC. Chemical treatment has selectivity against MC types, and it cannot mitigate the toxicity completely. Biological treatment can remove the bacteria and its toxin. Introducing a new bacterium that can degrade MC toxic may produce an unknown threat to the environment, or the new species can colonize the ecosystem and become a new threat to existing species. A new way to degrade MC is by using enzymatic degradation. Microcystinase A (MlrA) is an enzyme that is produced by some bacteria that can degrade MC. According to Liu et al., these bacteria strains were identified and found that MC degradation is not sufficient to mitigate the high

concentration of MC (ref). For that reason, cloning the gene into bacteria that can produce high MlrA concentration is this research focus.

2.1 MC characteristics:

MC gains attention because it is poisonous to humans and animals, and lower doses of MC cause cancer in humans (Wei et al., 2021). MC is a chemically stable molecule that can endure high temperatures and different pH levels, and for that reason, it resists traditional water treatments (Dexter et al., 2021; Li et al., 2017; Qin et al., 2019). Once cyanobacteria release the MC into the aquatic system, it exists for 2-3 weeks before the natural biodegradation process starts (David G. Bourne et al., 2001; Jones & Orr, 1994). MC is named according to the amino acids on positions 2 and 4. For example, MC-LR (Microcystin – Leucine Arginine) is named according to leucine (L) and arginine (R) located where reference figure 1. Cyanobacteria produced MCs to enhances their ability to live and establishing their colonies (Gan et al., 2012). MC is able to affect algae growth and makes a zone free of algae, so it eliminates the competition throughout the area the cyanobacteria colonized by using two methods. The first way is that the extracellular MC protects the bacteria from its competition in the ecosystem. Furthermore, intracellular MC enhances the cyanobacteria's photosynthesis process and makes it more resistant to environmental stress (De Figueiredo, Azeiteiro, Esteves, Gonçalves, & Pereira, 2004; Qin et al., 2019).

MC is synthesized through amicrocystin synthetase complex (mcy-genes) on the cyanobacteria, and it is an ATP-dependent reaction. It can be a nitrogen reserve for cyanobacteria and regulate protein synthesis. Many factors play a role in MC's production, like

light type, temperature, and nitrogen and phosphorus concentration (De Figueiredo et al., 2004). Some studies found that red light makes *M. aeruginosa* PCC7806 produce more MC, while blue light lowers the MC production rate (De Figueiredo et al., 2004; Kaebernick, Neilan, BöRner, & Dittmann, 2000; Rapala, Sivonen, Lyra, & Niemelä, 1997).

The temperature has an effect on the type of MC toxin to be produced, low temperature favoring MC-LR synthesis (De Figueiredo et al., 2004; Rapala et al., 1997). On the other hand, higher than 25C, the cyanobacteria will favor MC-RR synthesis (De Figueiredo et al., 2004; Rapala et al., 1997). Cyanobacteria that produce MC vary in response to the concentration of nitrogen and phosphorus. Some will be affected, like the non-nitrogen-fixing cyanobacterium *M. aeruginosa*. *M. aeruginosa* will be affected by the limited nitrogen while growing. Consequently, the effect is smaller than the usual cell size and higher intercellular MC production. *Microcystis* spp. and *M. aeruginosa* can produce more MC in higher phosphor concentrations (De Figueiredo et al., 2004).

2.2 MC-LR degradation:

The microcystin-degrading bacterium is a new strategy to eliminate MC in the environment. This method can be safe for the environment and can be done on a vast scale. Yang et al. were able to identify a new native bacterium called *Sphingopyxis sp*. YF1, with a high MC-degrading rate in lake Taihu. The degradation process starts with MlrA, which linearizes MC-LR by cleaving the peptide bond between Adda and arginine. The second enzyme, MlrB, produces tetrapeptide from the linear MC-LR. The third enzyme, MlrC, frees the Adda group from the tetrapeptide produced by MlrB or the linearized MC-LR produced by MlrA. The final enzyme in

the mlr cluster is MlrD, which is believed to be a membrane transporter enzyme that moves Adda through the bacteria cell wall. After the Adda group is freed from the tetrapeptide, phenylacetate (*paa*) cluster enzymes further degrade Adda to CO_2 in the TCA cycle (Yang et al., 2020).

Also, chemical treatment with MlrA is another method that is used to kill cyanobacteria in the water supply and degrade MCs. Treatment of cyanobacteria by Hydrogen Peroxide (H_2O_2) was questionable, but after adding MlrA to this treatment, both Cyanobacteria and Mcrosystien were eliminated, and the treatment has great potential. The treatment by H_2O_2 alone reduced the MC concentration in the water quickly by killing the bacteria. Still, the problem is that bacteria can enhance their ability to adapt to H_2O_2 and become resistant. Fortunately, MC-producing bacteria is less resistant to H_2O_2 (Dziga et al., 2019). After combining MlrA with H_2O_2, H_2O_2 will lysate the cyanobacteria. Within the lysate, MC-LR is presented, MlrA will cleavage the MC-LR. Also, they emphasize the temperature and the pH level MlrA can work in (Dziga et al., 2019).

Physical treatment has limitations in removing the MCs from water. Traditional water treatments like chlorination, activated carbon adsorption, and membrane separation can remove the cyanobacteria from the drinking water, but once the toxin is released into the water, these treatments cannot eliminate it (Liang et al., 2021). The current method uses Fenton-based technologies to eliminate MCs, and the drawback is that it is costly and the process consumes high energy (Liang et al., 2021).

2.3 MlrA characteristics:

One of the ways to remove MC is to use enzyme degradation. This pathway occurs naturally in the environment by some bacteria that are able to degrade MCs. From these bacteria, scientists found that there are four enzymes involved in MC degradation: MlrA, MlrB, MlrC, and MlrD (Qin et al., 2019). MlrA is believed to be a metalloprotease. The metalloproteases are a significant class of enzymes that cleavage peptides. These enzymes have metal ions in their active site, mostly is zinc ions. These ions stabilized the amino acids in the active site to attack the peptide carbonyl group in MC-LR and insert a water molecule. Still, two recent studies suggested that it is an intramembrane glutamate protease, and it belongs to type II CAAX prenyl endopeptidases (David G. Bourne et al., 2001; Dexter et al., 2021; Xu et al., 2019).

MlrA is identified as metallopeptidases based on inhibition by metallopeptidase inhibitors EDTA and 1,10-phenanthroline. Also, MlrA active sites are very similar to metalloproteases active sites. From the MlrA protein sequence, the region H260 AIH263 NE265 of MlrA contains the active sites with this sequence valine(a)-bXHbbHbEc (Bourne et al., 2001; Dexter et al., 2018). In figure 2b, MlrA was modeled using the phyre2 model, and it showed 100% confidence and 74% sequence coverage. Histidine and glutamic acid work by hydrolyzing the cyclic form of MCs and inserting a water molecule. Also, MlrA is able to affect MC-LR producing mcy genes and lower the transcription rate of MC-LR in the cyanobacteria. Hence, by reducing the MC production, the bacteria will decrease in numbers (Liu et al., 2020). MlrA initiates the MlrABC degradation pathway (Dexter et al., 2018) by linearizing MC-leucine-arginine (MC-LR). MlrA cleavage the peptide bond between Adda and arginine. This cleavage will remove around 60% of MC-LR toxicity (David G. Bourne et al., 2001; Dexter et al., 2018; Dexter et al., 2021; Kormas & Lymperopoulou, 2013; Liu et al., 2020; Yang et al., 2020).

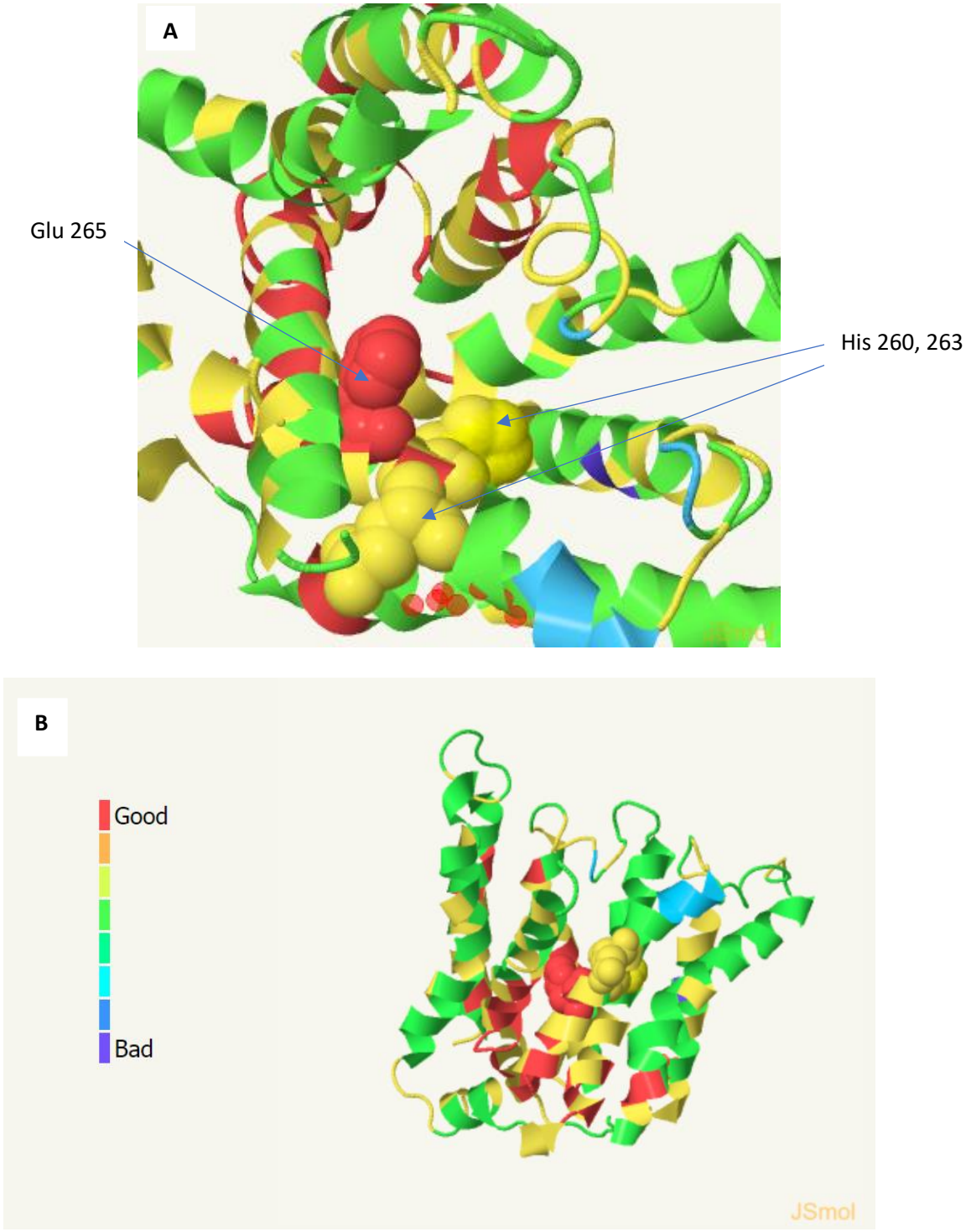

Figure.2: A: MlrA protein modeling showing the active site. B: showing the confidence in the modeling by Phyre2. Image by the author using (Kelley, Mezulis, Yates, Wass, & Sternberg, 2015).

While looking for a new way to enhance MlrA yield, a study done by Jiang et al. found that adding MC-LR to LB media while expressing the enzyme stimulates and increases the protein yields. They started with 0.4mg/l MC-LR added to LB media, and they saw a slight increase in *mlrA* gene transcription, which led to more protein yields. The higher increase in *mlrA* gene transcription was after adding 2mg/l MC-LR to LB media. This increase happened after 45minets of adding the MC-LR. This finding proves that the mlrA gene is responsible for detecting the cyanobacteria toxin and responding to its presents (Jiang, Shao, Wu, Xu, & Li, 2011).

Wu et al. immobilized MlrA by adding L-cysteine and Graphene Oxide to the enzyme and extended the lifetime of the enzyme. Consequently, increasing MlrA usability to degrade MC-LR (Wu et al., 2020). Liu et al. were the first team to reach more than 90% MlrA purity. MlrA is one of the most problematic proteins to purify (Liu et al., 2020); for example, MlrA is hard to fold correctly when expressed in the host cell (Liu et al., 2020). When expressed MlrA in the host cell, Liu et al. added a Maltose-binding protein tag (MBP) to MlrA. MBP-tag kept the protein soluble and reduced protein degradation. These properties make the MBP-tag an excellent candidate for obtaining the pure MlrA. The fused MlrA showed higher activity against MC-LR, RR, and YR than MlrA without MBP-tag (Liu et al., 2020).

Bourne et al. characterized mlr gene cluster in a study published in 2001. They stated that both MlrA and MlrC are homologous, and they are metalloenzymes. They were both repressed by EDTA but not MlrB. MlrD is a transporter enzyme that transfers the Adda group within the cell wall of the bacteria. Moreover, they have given the molecular weight for each enzyme product: linearized MC-LR weight 1012 Da, which is MlrA product, tetra-peptide weight 614

Da, which is MlrB product, but they did not identify MlrC product, which later described in the literature as Adda group (David G. Bourne et al., 2001).

In the literature on MlrA purification and expression, most methods need some stabilization to be attached to the enzyme. Either protein tag like Maltose Binding Protein tag or the enzyme needs to be immobilized like adding L-cysteine and Graphene Oxide to the MlrA. This finding raises a question in expressing the MlrA, is the protein tag play a massive role in its stability and activity?

2.4 Bacteria stress in this experiment:

In this experiment, Ni was added after adding the IPTG to the growth with Ni by 30 min. The reason for adding the Ni after 30 min is to ensure that the *E. coli* started the expression of the *mlrA* gene, and it is in the Log phase.

3. Chapter 3: MlrA expression and purification

3.1 Project objectives:

This study aimed to demonstrate the effect of adding nickel on the protein yield, demonstrate absorption and desorption of MC-LR to SPEEK membranes to use the membranes in future study, and investigate the activity of free MlrA.

3.2 Materials and Methods:

3.2.1 Strain and plasmid construction:

E. coli BL21 was purchased from New England Biolabs. The plasmid pET-21a (+), which has 5369 bp, was purchased from Novagen Life Technologies. Finally, a recombinant pET-MlrA plasmid, which has 6417 bp, was constructed and transformed into *E. coli* BL21. In this experiment, the 1048 bp *mlrA* gene was transferred to *E. coli* competent Bl-21with the heat shock method as part of the plasmid. The process started by using two tubes, one for pET plasmid containing 10 µl of 500mg/µl pET-21a plasmid. The second tube has 2 µl of *mlrA* DNA. Both tubes have 1.25 µl of BamHI 10 U/µL and XhoI 10 U/µL, which are restriction enzymes to cleave the pET plasmid and mlrA gene. Then, 17 µl water and 2.5 µl of 10X digestion buffer were added to both tubes. After incubation for 1 hour at 37°C, incubation for 5 minutes was done at 65°C to denature the enzymes.

By using 20 µl of recovery buffer, the cleaved gene and plasmid were recovered from the agarose gel. After retrieving the *mlrA* DNA and the plasmid, 10 µl of the plasmid, and 2 µl of *mlrA* DNA were incubated overnight with 1 µl of 5 U/µL T4 DNA ligase enzyme, 2.5 µl of 10x ligase buffer, and 1.5 µl of DI water. After the incubation, 5 µl of the ligation solution was added to 50 µl of media containing competent *E. coli* BL21. The *E. coli* and the *plasmid* were placed on ice for 30 minutes, then incubated for 10 seconds at 42°C then returned on ice for 5 min. Then 1ml of SOC outgrowth medium (New England, Biolab USA) was added to the tube containing the heat-shocked *E. coli* BL21. After mixing the *E. coli* with SOC media, the mixer was incubated for 2 hours at 37°C, and then 100 µl was spread on an (LB) agar plate containing 100 µl/ml of ampicillin at 37°C overnight.

3.2.2 Expression of MlrA:

LB media was used to grow the host bacteria to express the enzyme. A10 ml of LB media was used as an overnight culture, containing 10 μg/ml ampicillin, and was inoculated with a swap of one colony of *E. coli*. The overnight culture was incubated at 37° C with shaking at 200 rpm. Then, the overnight growth was transferred to 1L of LB media that contained 75 μg/ml ampicillin and was incubated at 37°C with shaking at 200 rpm until the optical density (O.D.) at 600nm reached 0.6-0.8. After reaching the OD at 0.6-0.8, 100 μg/ml, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the culture. For a culture with nickel sulfate, 1.4mM nickel was added after adding the IPTG for 30 min. After around 4 hours from adding the IPTG, cells were collected by centrifugation for 1 hour at 3750 rpm.

3.2.3 Cell lysate and protein purification:

After harvesting the cells, the cell pellets from 1L culture were resuspended in 10 ml of 5 mM sodium phosphate buffer, pH 7.4. The resuspended cells were sonicated at low amplitude using 5-second pulse and 5-second rest cycles for 15 min in an ice bath. The supernatants were then centrifuged for 1 hour at 3750 rpm, a second centrifuge process for 10 min at 13000 rpm was done. The lysate filtration was made by a 0.22 μm syringe filter. Furthermore, the purification was done using the Fast Protein Liquid Chromatography (FPLC) system from ÄKTA. The column, which was from GE Healthcare-Bio-Sciences, was cobalt loaded Hi-Trap Immobilized Metal Affinity Chromatography (IMAC) column to purify the 6x His tagged MlrA protein. Histidine had an imidazole ring that would bind to the metal ions of the column, in this case, cobalt. The sodium phosphate buffer had 250mM imidazole with pH=7.3. After running the

buffer in the FPLC, the imidazole would compete with the protein and replaced it in the column. The elution would have MlrA. The fractions collections process was set up to have 5 ml of the eluted buffer in each fraction, and the column size was 5 ml. However, before going further, buffer exchange would be made by using a low salt buffer, in this case, 5 mM sodium phosphate buffer, 15 ml for every 5 ml fraction. After buffer exchange, protein fractions would be subject to sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE).

3.2.4 Concentration determination:

The concentration of the MlrA was determined by using a derived form of the Beer-Lambert Law. The molecular weight and extinction coefficient were determined from the MlrA sequence, which was

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mrefvkqrpl lcfyalaili altahalram sptplgpmfk mlqethahln iitavrstfd 61
ypgaytlllf paapmlaali vtgigygrsg frellsrcap wrspvswrqg vtviavcfla 121
ffaltgimwv qtylyappgt ldrtflrygs dpvaiymmla aslllspgpl leelgwrgfa 181
lpqllkkfdp laaavilglm wwawhlprdl ptlfsgepga awgvivkqfv iipgfiagti 241
iavfvcnklg gsmwggvlih aihnelgvnv taewaptvag lgwrpwdlve favaiglvli 301
cgrslgaasp dnarlawgnv ppklpggatd ksgana
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From the sequence, MlrA had 8 tyrosine, 13 tryptophan, and 5 cystine. By using this formula $Extinction\ coefficient\ of\ protein = (nW\ x\ 5500) + (nY\ x 1490) + (nC\ x\ 125)$, the extinction coefficient would be determined. Also, the protein absorbance at 280nm was measured using Shimadzu UV-2600 Spectrometer.

3.2.5 Preparing samples for metal analysis:

Inductively coupled plasma mass spectrometry (ICP-MS) was used for analyzing nickel and zinc on the protein. The sample was diluted by 1 to 10 dilution factors using dilute acids. For example, 1 ml of concentrated MlrA + 9 ml of 2% nitric acid. A dilution factor from 10 to 50 was usually acceptable for liquid protein (Wilschefski & Baxter, 2019). ICP-MS was used to determine the metals present on the enzyme, for example, nickel, zinc, and to compare enzymes from different growth conditions.

3.2.6 MlrA activity experiment:

For the enzyme activity assay, MC-LR was treated with MlrA, and the activity was checked at three intervals of 0, 4, and 24 hours. 1325 μL 5mM sodium phosphate buffer was added to a 20 ml glass vial, then 475 μL of MlrA enzyme was added. Finally, 200 μL MC-LR solution (1 mg/L) was added as the final step of the reaction. The final volume was 2 ml with a final concentration of 0.216 mg/ml of MlrA in the solution. The reaction was stopped by the addition of 50 μL 5% acetic acid to 500 μL of the solution at 0, 4, and 24 hours. After collecting the samples, they were analyzed by High-performance liquid chromatography (HPLC).

3.2.7 FTIR for SPEEK membranes:

SPEEK offers high proton conductivity (Daud et al., 2021), and MlrA was a positively charged enzyme. The enzyme was attached to the SPEEK membrane to be used on the enzyme

activity as an immobilized enzyme. Fourier transform infrared (FTIR) was used to detect the presence of the MlrA enzyme on SPEEK membranes after immobilization.

3.2.8 Absorption and desorption of MC-LR to SPEEK membranes:

MlrA was immobilized to SPEEK membranes followed by incubation with MC-LR the same as other experiments. Therefore, this experiment was designed to confirm the absorption of MC-LR to the membrane and to try to desorb it back from the membrane. 200 µL MC-LR (10 µg/L) was added to 1800 µL phosphate buffer (5 mM). All was added over a control SPEEK membrane. Before taking the sample after 24 hours, 667 µL methanol was added. Like the free MlrA experiment, samples were taken after 0, 4, and 24 hours.

3.3 Result and discussion:

3.3.1 Cloning results:

Figure 3 shows the plasmid on lines 2 and 3, which had 5403 bp, and the *mlrA* gene, which had 1048 bp, on lines 5, 6, and 7, which was used as described on the plasmid construction step. After the cloning was done, plates containing ampicillin showed colonies indicating the successful cloning of the plasmid pETMlrA, which had 6451 bp into the *E. Coli*.

Figure.3: lane 1) standard DNA Ladder starting from 4000 bp to 100 bp. Lane 2) pET plasmid uncut 5443 bp. Lane 3,4) pET plasmid cleaved 5403 bp. 5,6,7) mlrA gene cleaved 1048 bp.

3.3.2 Purification results:

A gradient of 250mM imidazole buffer was used. At 62.5mM imidazole, the majority of MlrA was eluted. At 125mM imidazole, a small peak was formed; these fractions had a lower MlrA concentration. At 187.5mM and 250mM imidazole, low or no trace for MlrA was found.

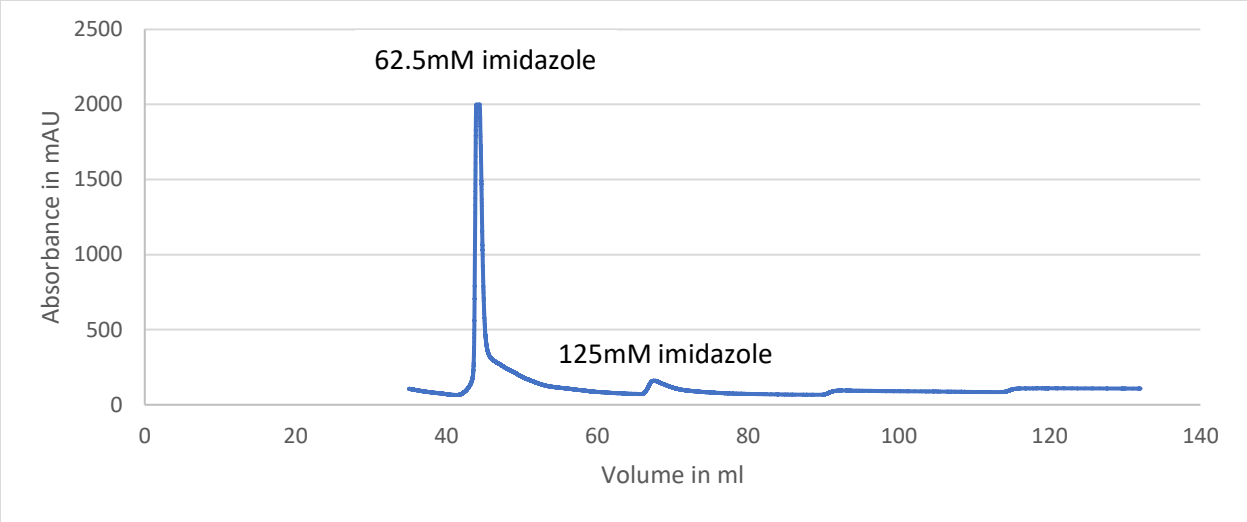

Figure.4: FPLC graph showing the peaks from which the fractions were collected. MlrA was eluted after 43ml until around 75ml. The peak at 43ml was using 62.5mM imidazole, and the peak around 73ml was using 125mM imidazole.

3.3.3 SDS-PAGE result:

Results showed a band at around 42 kDa to indicate the presence of MlrA (Figure 5a). The arrows indicate the band that formed from the fractions that were selected. The higher the concentration of imidazole, the more enzyme purity obtained. Fractions 5, 6, and 7 were from 62.5mM imidazole, and they showed a higher enzyme presence than what was presented at fraction 10 from 125mM imidazole.

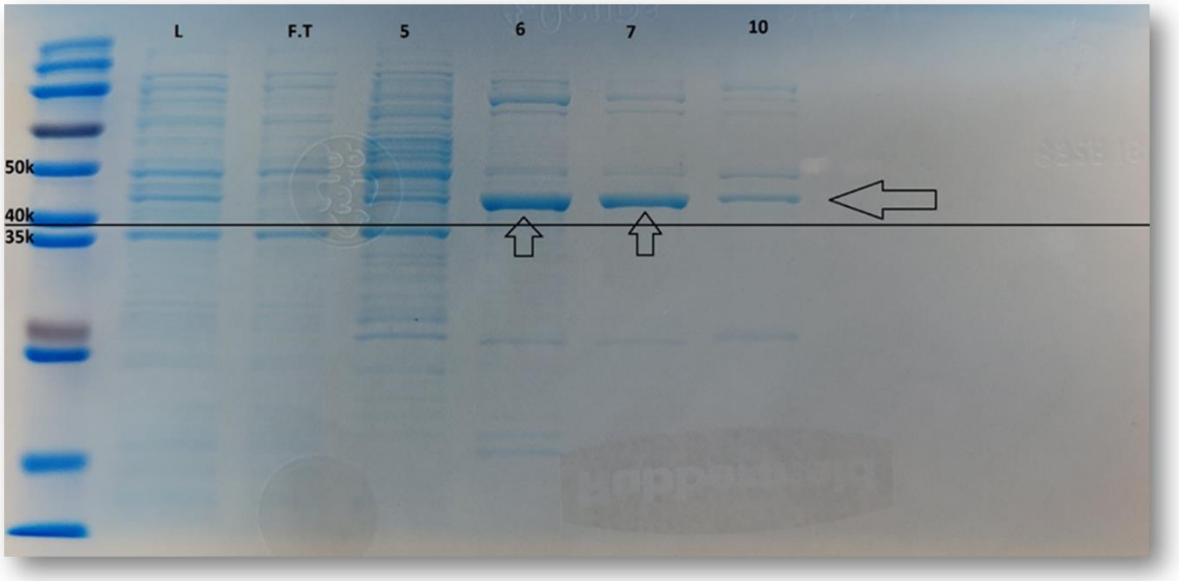

Figure.5a: SDS-PAGE gel showing the bands with MlrA around 42 kDa from the fractions from the FPLC. L=Lysate, F.T.= Flow-through, 5, 6, 7, and 10 are the fractions numbers.

For the Ni growth, MlrA was expressed at a higher yield and higher concentrations (Figure 5b), which indicated that the bacteria were indeed under the stress of the added Ni. After three experiments, the average enzyme concentration was higher when Ni was added. The average concentration for Ni growth is 1.26 mg/ml. In comparison, the normal growth was averaged around 1.097 mg/ml from the same volume. MlrA bands were clearly visible in the SDS-PAGE gel, especially in 125mM imidazole lane 11. This clarity was noticeable when comparing lane 10 from figure 5a with lane 11 from figure 5b. Table 1 shows the concentration from each growth, and the MlrA concentration was the average of the three experiments. MlrA expression with Ni had the higher concentration. Even though the differences were not significant, the concentration on MlrA yield was higher in every growth with Ni.

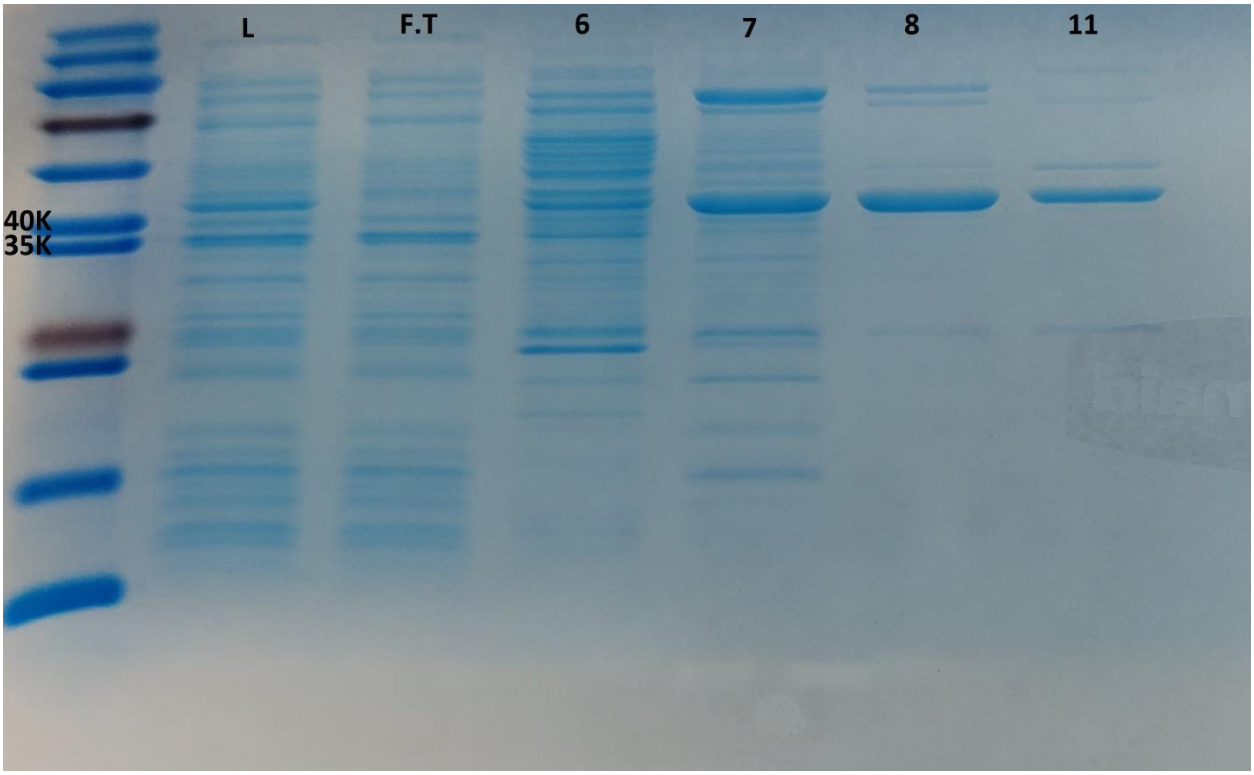

Figure 5b: MlrA from growth with Ni. Lanes 7 and 8 are from 62.5mM imidazole, lane 11 is from 125mM imidazole.

Table 1: showing the individual concentration for each growth. Fractions from 125mM imidazole were not included in the experiments averaging.

Growth volume	MlrA concentration
1L MlrA	0.892 mg/mL
1L MlrA +Ni	1.16 mg/mL
1L MlrA	1.17mg/ml
1L MlrA+Ni	1.24 mg/Ml
1L MlrA	1.23 mg/mL
1L MlrA+ Ni	1.38 mg/mL

3.3.4 MlrA activity result:

After confirming the presence of MlrA using SDS-PAGE, 5mM sodium phosphate buffer was used for the buffer exchange and to concentrate MlrA. The activity experiment was done as described in the methods section. MlrA showed the ability to degrade MC-LR after 24 hours. A new peak was shown at m/z 1010.1 on the HPLC figure 6a, which was not shown in the control experiment of figure 6b. Cyclic MC-LR was at m/z 995.6, while linearized MC-LR was at m/z 1010.1. Also, it was clear that not all the MC-LR was degraded, which suggests that the enzyme needs more time to degrade the toxin. As described, the samples were taken at 0, 4, and 24 hours. Only the sample at 24 hours showed the linear MC-LR. The experiment with the enzyme grown with added Ni showed that the enzyme was inactive even though the enzyme had a higher concentration (1.16 mg/ml) than the regular enzyme, which was 0.892 mg/ml. Because the graph of the activity experiment with added Ni was similar to the control, the graph of the activity experiment with added Ni was not shown.

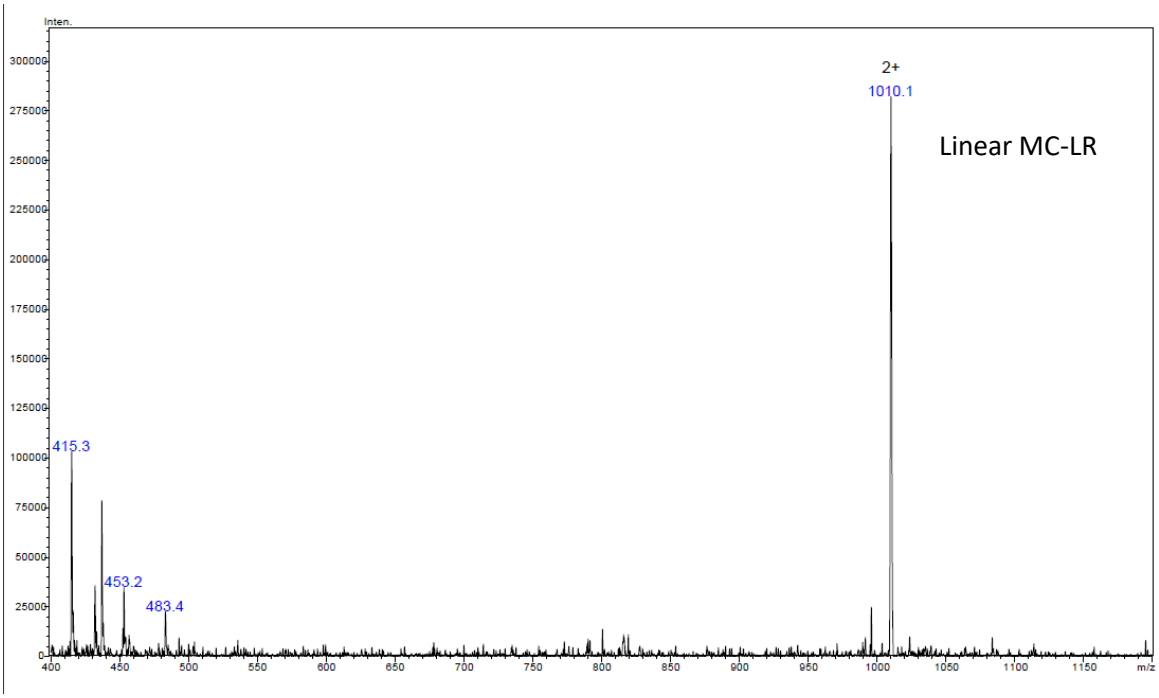

Figure 6a: Electrospray ionization (ESI) MS analysis of the cyclic MC-LR. After 24hrs of the treatment with the MlrA enzyme, linearized MC-LR was produced at m/z 1010.1 (5.91 mint MS run).

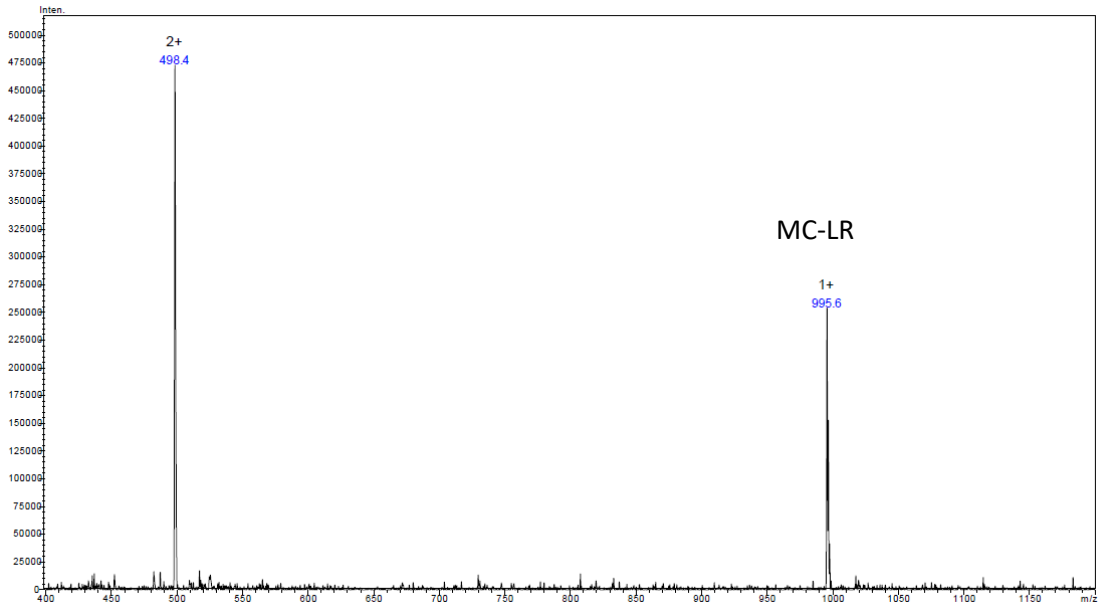

Figure 6b: The control was only MC-LR and 5mM sodium phosphate buffer. The control run that only showed MC-LR at m/z 995.6.

3.3.5 FTIR results:

From figure 7, at around 1700cm-1, carbonyl stretch was present in both the control (blue graph) and the SPEEK_MlrA membranes (orange graph), but the intensity of the stretch was lower in SPEEK_MlrA around 1750cm-1. Additionally, before 3000cm-1, there was a saturated CH stretch from around 2750cm-1 to around 3600cm-1, the IR was absorbed by the SPEEK_MlrA more than the control SPEEK, indicating that the enzyme is attached to the membrane. Generally, the membranes had similar absorbance behavior.

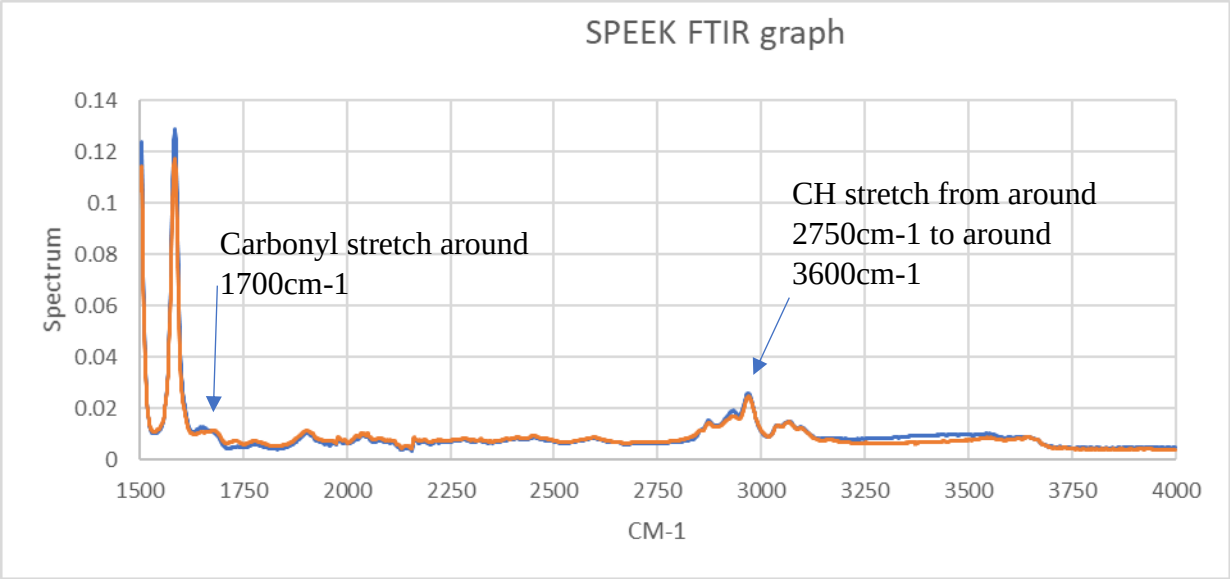

Figure 7: showing FTIR graph of SPEEK_control without MlrA in blue and showing SPEEK_MlrA in orange.

3.3.6 Absorption and desorption of MC-LR to SPEEK membranes results:

The results showed that adding methanol to the membrane was successfully desorb MC-LR. At 0 hours, figure 8a showed the toxin peak at around 5 min. Figure 8b showed how the SPEEK membrane absorbed the toxin. The HPLC reading was lower than the reading from 8a. Figure 8c shows how methanol desorbs MC-LR from the membrane. The peak returned to its expected peak.

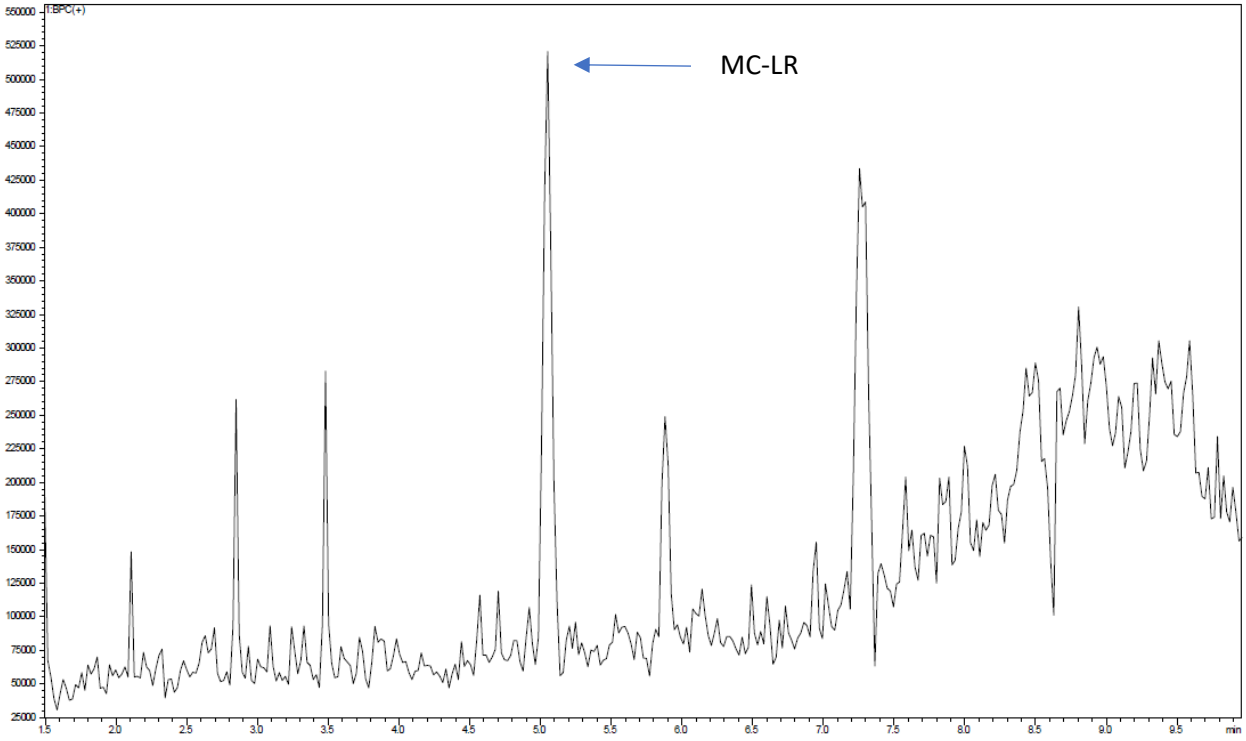

Figure 8a: At 0 hours, there is no absorbance to the SPEEK membrane.

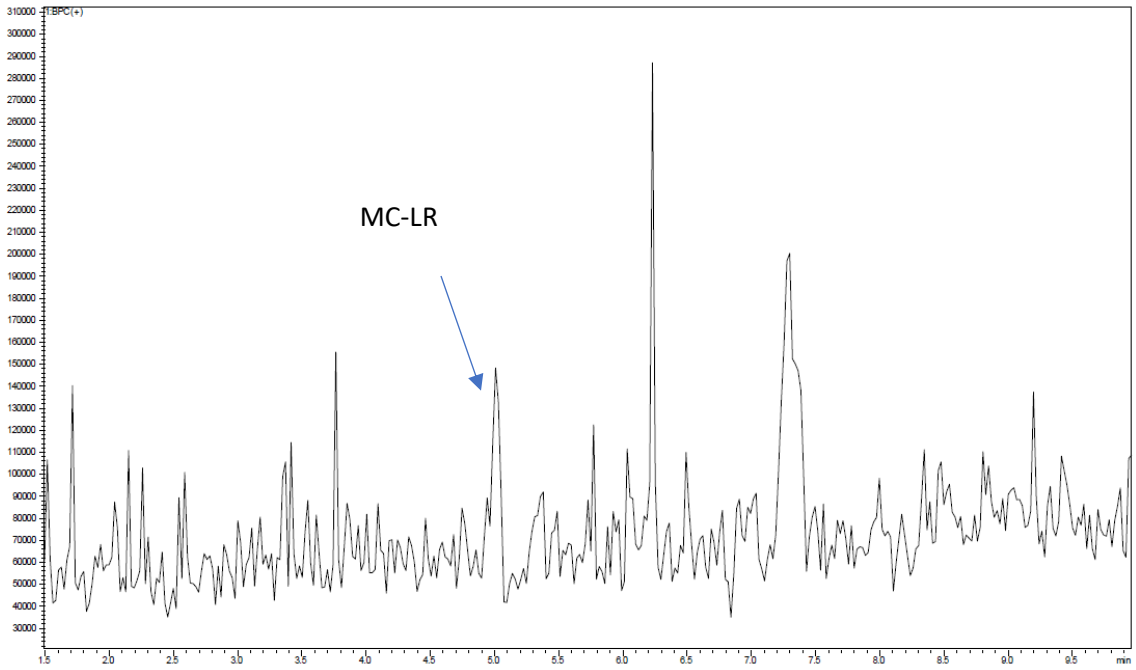

Figure 8b: At 4 hours, a small peak at 5 min indicates that MC-LR was absorbed by the SPEEK membrane.

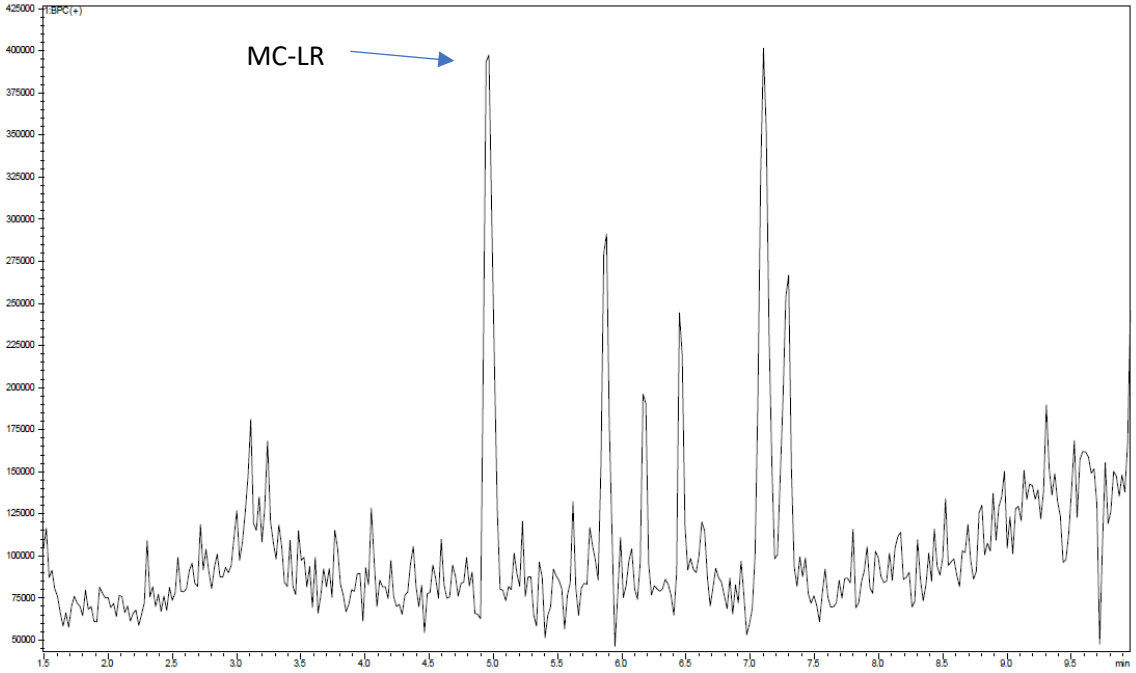

Figure 8c: At 24 hours shows that the MC-LR is desorbed from the SPEEK membrane.

3.3.7 Metal analysis results:

Because MlrA has a zinc-binding site, zinc concentration was higher than the nickel in all experiments. When buffer exchange was done, it was made by three times the volume of the samples to make sure to remove any residues in the samples. From table 2, nickel presence was high in samples from growth with Ni, and Ni existed in low concentration in non-Ni growth. Zinc concentration was high regardless of the growth type, and it correlated to the enzyme concentration. It is clear that the Ni did not substitute the Zn. From the MlrA activity experiment, the enzyme with the addition of Ni was not active. That may indicate that the Ni was attached to the enzyme on the active site directly like a competitive inhibitor, or it may act as a non-competitive inhibitor to MlrA and attached on another site, and by attaching to a different site, Ni change the three-dimensional structure, and the enzyme becomes inactive.

Table 2 Concentration of Zn and Ni in MlrA samples.

Sample concentration	Ni	Zn
MlrA +Ni = 1.16 mg/ml	202.2ppm	581.3ppm
MlrA Con= 0.892 mg/ml	33.8ppm	504.2ppm
MlrA Con=0.411 mg/ml	34.02ppm	127.9ppm

3.4 Conclusion:

In this study, an active MlrA enzyme was produced from a heterologous host, and the enzyme was able to degrade MC-LR and convert it to linearized MC-LR. Also, adding nickel to LB media increased the protein yield in *E. coli*. Even though the enzyme after adding nickel was not active, non-metalloenzyme could benefit from this method in the future. Additionally,

investigating how to desorb MC-LR from SPEEK membranes can help in the future with the experiment of immobilized MlrA to SPEEK membrane.

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Appendix

Investigating Microneedles releasing High Molecular Weight Proteins.

Abstract

This study was to investigate poly (vinyl alcohol) (PVA) as a microneedle to release a large molecular weight protein using PspA protein as a drug model. The release from the microneedles was successfully obtained, confirming a controlled release of PspA protein over time. From the successful release of the PspA protein, a similar weight protein can be used.

1. Introduction on PspA:

According to the CDC, pneumococcal diseases occur worldwide, especially in low-income countries where vaccines are limited or unavailable. PspA, a surface protein of *Streptococcus pneumoniae*, is the next generation of pneumococcal vaccines (Baril et al., 2006). PspA is an antigen that provides stimulation to the immune system to fight pneumococcal diseases. Also, the PspA vaccine will protect against pneumococci of more than one capsular type (L. S. Mcdaniel, Sheffield, Delucchi, & Briles, 1991). PspA is naturally expressed on the surface of most pneumococci types, and it helps the pneumococci to colonize the respiratory tract. pspA gene has variable sequences in different pneumococci. Because of the gene variations, PspA protein has different amino acid sequences and molecular weights (Baril et al., 2006).

2. Incorporating PspA in microneedles and microneedles fabrications:

Streptococcus pneumoniae is a gram-positive bacterium. Hence, like many gram-positive bacteria, *S. pneumoniae* infections are hard to be treated (J. Jedrzejas, Ejvis Lamani, & S. Becker, 2001). *S. pneumoniae* colonizes the upper raspatory tract in humans (J. Jedrzejas, 2001; Moore, Bosarge, Quin, & McDaniel, 2006). Also, it affects more than 100,000 people in the United States each year, causing more than 40,000 deaths from this infection, especially in the elder population (Briles et al., 1998; Moore et al., 2006). In developing countries, mortality from *Streptococcus pneumoniae* is usually in children of five years or younger. PspA is found on the surface of all pneumococci bacteria. The function of PspA is to help the *pneumococci* colonize and affect the host (Moore et al., 2006).

Developing vaccines can be done by using the same antigen that causing the disease. The vaccine can stimulate the immune system to identify the antigen and develop an antibody against it. PspA is what makes the *Streptococcus pneumoniae* attached to human lactoferrin and interferes (Baril et al., 2006). By administering the PspA protein as a vaccine, PspA will stimulate the host to develop an immune response, making the body develop antibodies to *Streptococcus pneumoniae* (Baril et al., 2006). There are 5 clades for the PspA from 2 families. Hence, the variation in the PspA protein molecular weight. From the 5 clades, clade 2 from family 1, 3, and 4 from family 2 protects against most pneumococcal strains (Baril et al., 2006). The alpha-helix domain of PspA can trigger the protection against *Streptococcus pneumoniae*; from this alpha-helix domain, a fragment of around 38 kDa is used in many studies (Aljewari et al., 2020).

Due to the degradation or low absorption in some drugs, some drugs have to be administered intravenously or subcutaneously if taken orally. The conventional injection can

deliver drugs through the skin, but this method is limited to lipophilic and low molecular weight drugs (Gill & Prausnitz, 2007). Hence, a low-cost and safe method of administering drugs should be developed. Pathological science has shown that the skin is full of immune cells and scientists now know that this is a suitable area for the application of vaccines and other drugs. The pharmaceutical industry is trying the outstanding possibility of using microneedles, and they are working on a new process to turn the whole concept of microneedles into a commercially viable reality. Microneedles can be developed using different materials like Polyvinyl alcohol (PVA), plastic, and stainless steel. PVA was chosen because of its biocompatibility, and PVA is non-carcinogenic (Kamoun, Chen, Mohy Eldin, & Kenawy, 2015).

Microneedles were first introduced in the 1970s, and it was handmade. In the late 1990s, with the nanotechnology advancements, microneedles gained more attention by scientists as a way to deliver drugs that required a direct injection to the bloodstream due to its low absorbance in the digestive system and without puncturing the skin like conventional needles (Kim, Park, & Prausnitz, 2012; Prausnitz, Mitragotri, & Langer, 2004). Many types of microneedles were introduced, like coated microneedles, hollow microneedles, and hydrogel microneedles (Gill & Prausnitz, 2007). Also, microneedles can eliminate the high cost of qualified staff to administrate the microneedles since they stack to the skin and do the release. This cost-saving is essential in low-income countries and countries with limited health care infrastructure.

One type of microneedles is the premade microneedles. These microneedles can be coated with a drug to be administered to the skin (Gill & Prausnitz, 2007). The downside of this method is that the microneedles can lose the drug during administration. Therefore, the coating process can lower the sharpness of the tip and waste materials because of the added coating

layers (Gill & Prausnitz, 2007). Also, the microneedle that was used in Gill and Prausnitz's study was made of stainless steel.

Another type is hollow microneedles. Those made with silicon are currently in use in healthcare monitoring technologies, and the Food and Drug Administration (FDA) has granted its use (Li et al., 2019). One of the properties of silicon-based microneedles is their ability to penetrate the skin and not break inside. Nanosensors can be fitted inside Hollow microneedles that are silicon-based, which monitor the individual health for a long time without irritation.

Microneedles are fabricated with different materials, stainless steel, plastic, silicon. However, a low-cost and improved property alternative can be found in polymeric microneedle (Castilla-Casadiego et al., 2021). Polymeric microneedles can control the drug release over time with the microneedle degradation in the skin. PVA and chitosan are examples of polymeric microneedles. They are both biocompatible, non-toxic, and relatively low-cost (Aljewari et al., 2020; Castilla-Casadiego et al., 2021).

PVA gels that were crosslinked with glutaraldehyde can absorb water because of their high degree of swelling in an aqueous solution (Agarwal, Alam, & Gupta, 2013). This absorption causes the PVA gel to control the release of the protein incorporated in the gel in hydrophilic buffers (Aljewari et al., 2020). This property of PVA gel makes it an excellent candidate for using it in many studies (Aljewari et al., 2020). Furthermore, PVA gels can make the drug completely available to be discharged. Also, PVA is non-biodegradable, which makes PVA stable when releasing the drugs for a long time.

After isolating the pspA gene, it was cloned into a heterologous host, in this case, *E. coli*. After the enzyme expression, PspA protein was used as a vaccine. PspA protein showed its

ability to prevent many infections caused by *S. pneumoniae* (Briles et al., 1998; Moore et al., 2006; Saumyaa et al., 2016). PspA is a significant protein, and its molecular weight can go up to 130kDa (L. Mcdaniel et al., 1997). Hence, fragments from PspA protein were used in many studies instead of the whole protein, including this study. The goal of this study is to confirm the release of the PspA vaccine via microneedles. The microneedles were fabricated using Polyvinyl alcohol (PVA). PVA is biocompatible; as a hydrogel, PVA is used in many medical and engineering applications. PVA can be dissolved in water and can be crosslinked using Glutaraldehyde (GA) (Sana Ullah et al., 2020). GA is a known agent for crosslinking polymers. PVA, as a crosslinked hydrogel, can not dissolve in water, making it an excellent candidate for microneedles fabrication (Aljewari et al., 2020).

Microneedle fabrication:

3. Project objectives:

Investigate PVA microneedle as an ideal carrier for large proteins by using PspA as a drug model.

4. Microneedle fabrication with PspA:

4.1 Material and methods:

The hydrogels used for this study were prepared using poly (vinyl alcohol) PVA (98% hydrolyzed, the molecular weight of 70,000 - 100,000 g/mol−1), acetic acid (AA), and glutaraldehyde (GA) (50% aqueous solution). All materials for the formation of membranes were purchased from Sigma-Aldrich chemical company (St. Louis, MO, USA). Ampicillin was also

purchased from Sigma-Aldrich chemical company. Sodium phosphate and Luria broth were purchased from Fisher Scientific (ThermoFisher Scientific, USA). All chemicals were used without further purification and were of analytical grade. The alpha-helical domain of PspA was purified from the *E. coli* clone.

4.2 Growth and purification of PspA:

A10 ml of LB media was used as an overnight culture, containing 10 μg/ml ampicillin, and was inoculated with a swap of one colony of *E. coli*. The overnight culture was incubated at 37° C with shaking at 200 rpm. Then, the overnight growth was transferred to 1L of LB media that contained 75 μg/ml ampicillin and was incubated at 37°C with shaking at 200 rpm until the optical density (O.D.) at 600nm reached 0.6-0.8. After reaching the OD at 0.6-0.8, 100 μg/ml, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the culture. After four hours of the induction with IPTG, bacteria were collected and centrifuged for one hour at 3750 rpm at 4°C. Pellets were resuspended with 10ml of 10mM sodium phosphate buffer at 7.4 pH for each one-liter growth broth. The cells were sonicated for 15 min, 5 seconds pulse, 5 seconds rest at 20% power in an ice bath. After the sonication, the lysate was centrifuged for one hour at 3750rpm at 4°C. Then, the microcentrifuge was used to separate the lysate further. The microcentrifuge setting was 13000rpm for 5min. Affinity chromatography (FPLC) was used to purify PspA. SDS-PAGE was used to confirm protein purity and the PspA molecular weight, which is 37kDa.

4.3 Microneedles with the PspA protein:

Microneedles were fabricated by adding 5g of PVA powder to 40ml of distilled water at boiling temperature. Then, 960µl of PVA and 250 µl of PspA was added to a small vial which contained a Stirring bar to mix the solution, 108.75µl of 10% sulfuric acid was added as a catalyst, 36.25µl of 10% acetic acid was added as a buffer, 36.25µl of 50% methanol was added as a reaction stopper. Finally, 108.75µl of 1% GA was added as a crosslinker. After 15 seconds, the mixer was brewed to prefabricated microneedle molds, and the molds were left for 6 hours to crosslink at room temperature.

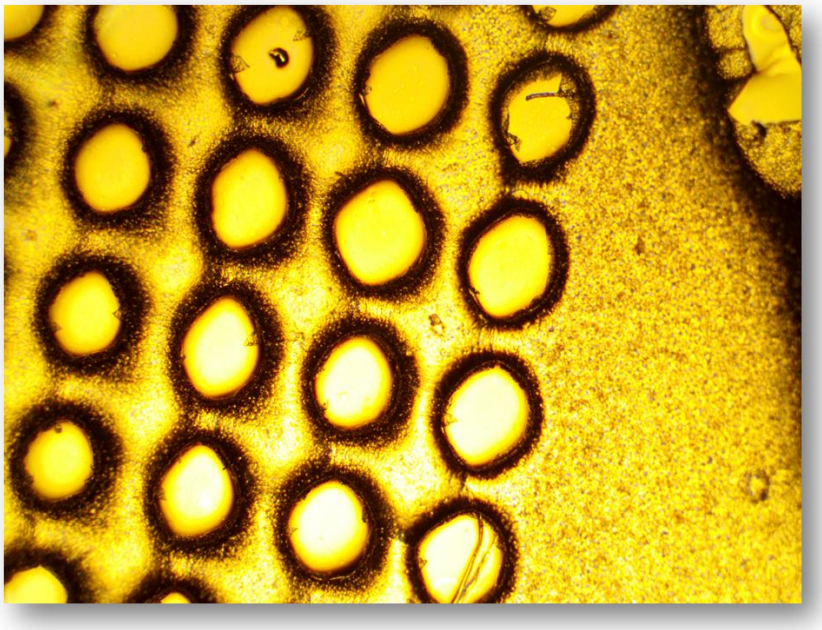

Figure 7: Microneedle after it was made under the light microscope.

4.4 The diffusion cell experiment:

Microneedles were placed between the three diffusion cells. The diffusion cells were filled with 5ml buffer. The buffer was stirred all the time, and the temperature was kept at 37 °C. 1ml of the buffer was checked every 30 minutes with the UV-Vis to determine how much protein was released. After checking the O.D., the buffer was returned to the cell. The absorbance was checked at 277 nm.

5. Result:

5.1 Microneedle fabrication:

Three microneedles were made for every experiment containing the PspA. The biggest challenge was to cast the microneedles without bubbles. From figure 8, bubbles were easy to form, so blowing air was one of the solutions to remove them from the microneedles before crosslinking.

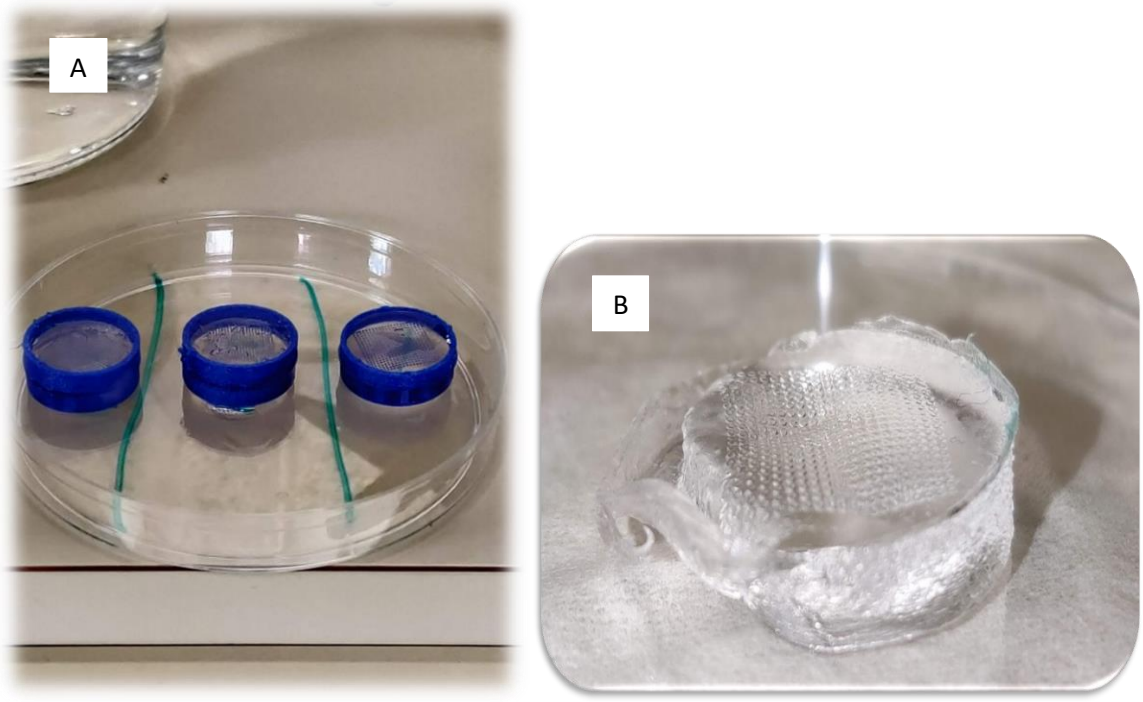

Figure 8a: showing three microneedles in their molds. 8b: showing a microneedle after the experiment.

5.2 Diffusion cell experiment result:

Each microneedle was placed between two cells. One of the cells was filled with a buffer like what is shown in figure 9.

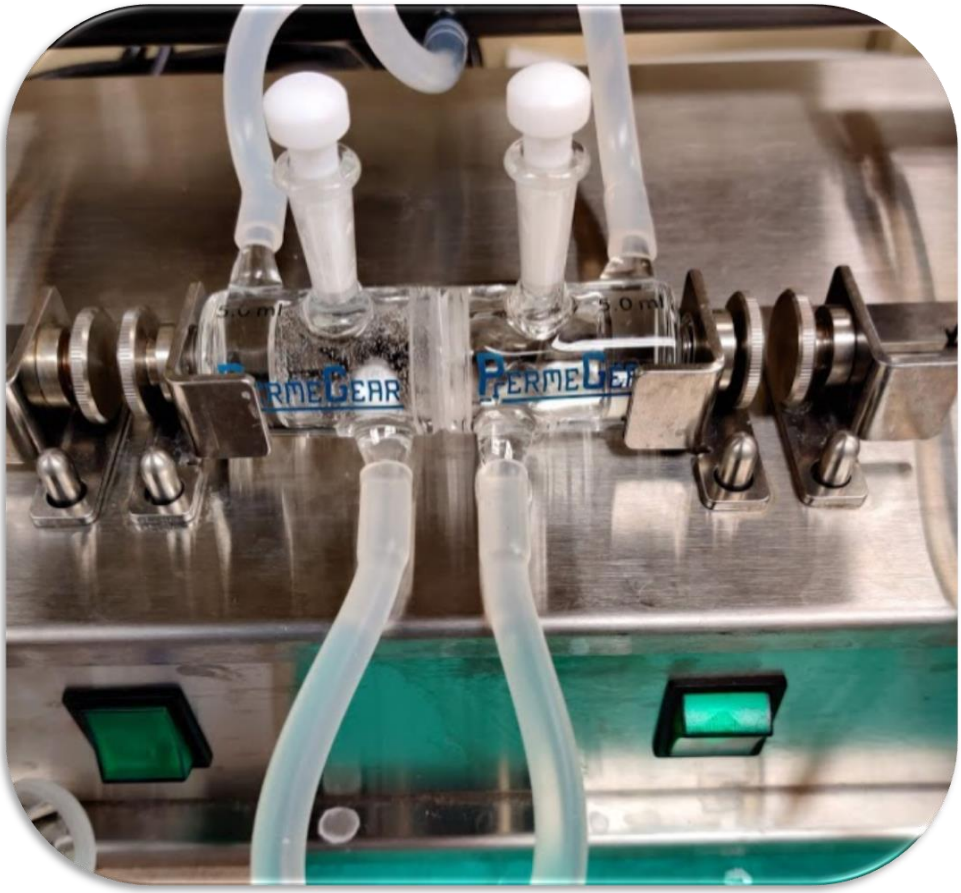

Figure 9: The diffusion cells when the experiment was running.

The absorbance was measured every 30 minutes. Figure 10 below gives the average of the three runs, proving the release of PspA into the buffer. From the figure, the release was increasing with time, and the experiment was designed to run for two hours only, and it had terminated after 2 hours. PspA concentration after 30 min was 0.001 mg/ml, and after 2 hours, the concentration was 0.0033 mg/ml. The starting concentration in the microneedles was 0.12 mg/ml. The release was around 3% of the PspA in the gel in 2 hours. In comparison to Swiatlo's et al. study, the released protein is higher in this study than what Swiatlo used to vaccinate the

mice in their study (Swiatlo, King, Nabors, Mathews, & Briles, 2003). They used 0.0005 mg/ml of PspA protein, and the release from this study's gels is 0.0033 mg/ml.

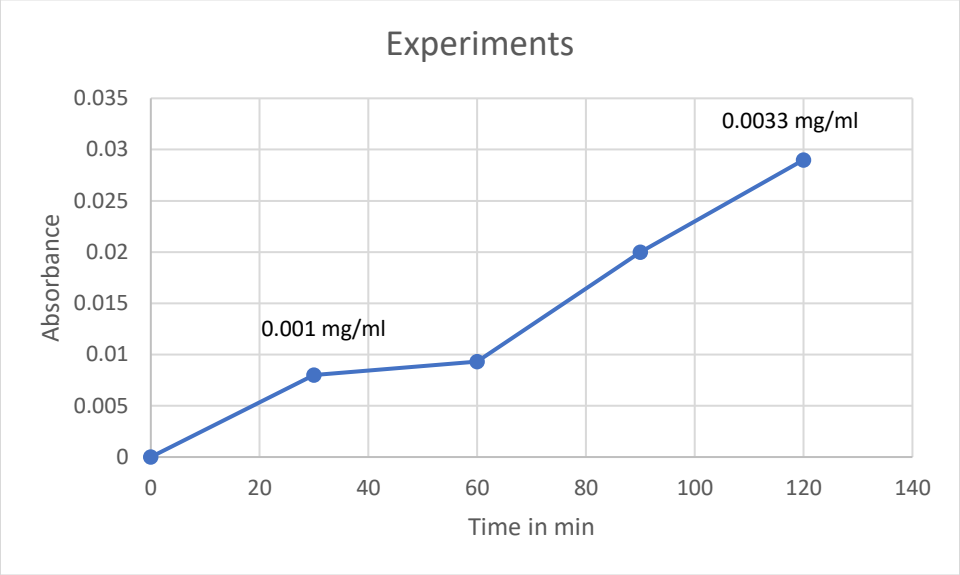

Figure 10: The diffusion graph demonstrates a constant release until two hours mark.

6. Conclusion:

In this study, microneedles have proven to be suitable for drug delivery. PspA had a significant molecular weight, and it was diffused through the microneedles, which means microneedles are one of the new modern techniques for drug delivery. This technique can be used in the future for vaccines delivery as a cheap and safe alternative to regular needles. Also, microneedles can be used for similar molecular weight vaccines. Also, for future work, making layers of membranes on top of each other where the lower layer has the drug, and the top layer act like a protector and cover can increase the drug release to the buffer in diffusion cell at the

same time. Furthermore, using a centrifuge when making microneedles can help concentrate the drug in the tips of the microneedle and accelerate the release.

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