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Second cycle 30 credits

Development of a method to detect lysis and investigation if ozone has a lysing effect on *Escherichia coli*

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Development of a method to detect lysis and investigation if ozone has a lysing effect on *Escherichia coli*

Master thesis in Medical Biotechnology Klara Andrup

KTH Royal Institute of Technology In collaboration with Sangair AB

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Abstract

This project was conducted at Sangair. The company is currently developing a medical device aimed at treating bacteremia with ozone. Bacteremia is a condition that occurs when bacteria enters the bloodstream, and can trigger sepsis and septic shock, potentially leading to death if untreated. Antibiotics have traditionally been the way to treat bacteremia, but the looming threat of antibiotic resistance worldwide threatens this way of treatment, and research into novel approaches to eradicate the bacteria needs to be done. As part of Sangairs development, an investigation was done to see how ozone interacts with bacteria. The project aimed to 1) develop a method to detect bacterial lysis and 2) use the method to detect if ozone had a lysing effect on the bacterial model organism *Escherechia coli.* To test the method, the BL21 strain of *E. coli* was transformed with the pUC-19 plasmid to produce the reporter enzyme Beta-galactosidase. The Beta-galactosidase activity was then measured in a supernatant of sonicated bacteria, which confirmed the suitability of the method to detect bacteria cell lysis. To be able to see if ozone had a lysing effect, while still being able to measure Beta-galactosidase, it was found that the optimal setup for this was using an ozone concentration of 6 g/m^3, a gas flow of 5 ml/min and a liquid flow of 25 ml/min. The results acquired with this setup indicated that ozone had a lysing effect on *E. coli* but more studies are needed to verify this. It was further investigated if Beta-galactosidase detection could be improved by addition of bovine serum albumin (BSA) to quench residual ozone in the samples, but the results showed that it did not have any effect on the Beta-galactosidase enzymatic activity. As a final experiment, endotoxins that were released upon treatment were also measured, and it was found that when bacteria are treated with ozone, lipopolysaccharides (LPS) and peptidoglycans are released, further confirming lysis of the bacterial cells.

Keywords

Bacteremia, Ozone, Sepsis, Septic shock, Antibiotic resistance, Lysis, Beta-galactosidase, Sonication, ozone concentration, Bovine serum albumin (BSA), Endotoxins

Sammanfattning

Detta projekt genomfördes på företaget Sangair som för närvarande utvecklar en medicinsk utrustning för att behandla bakteriemi med ozon. Bakteriemi uppstår när bakterier hamnar i blodomloppet, vilket kan trigga sepsis och septisk chock, med potentiellt dödligt utfall om obehandlat. För närvarande används antibiotika för att behandla bakteriemi, men det ökande hotet med antibiotikaresistens världen över innebär att forskare behöver hitta nya vägar för att behandla bakterierna. Som en del av Sangairs utveckling gjordes en undersökning för att se hur bakterier påverkas av ozon. Projektet syftade till att 1) utveckla en metod för att detektera bakterie-lysis och 2) använda metoden för att se om ozon hade lyserande effekt på model-organismen *Escherichia coli*. För att testa metoden transformerades stam BL21 av *E. coli* med en pUC-19 plasmid för att producera beta-galaktosidas. Proteinets aktivitet mättes sedan i sonikerade bakterier, vilket visade att det var en effekitiv metod för att lysera bakterier och sedan mäta aktiviteten. För att undersöka om ozon hade en lyserande effekt, och samtidigt bestämma om man kunde mäta protein fann man att det optimala experimentella upplägget var att använde en ozonkoncentration på 6 g/m^3, ett gasflöde på 5 ml/min och ett vätskeflöde på 25 ml/min. Resultatet från studien indikerade att ozon har en lyserande effekt, men fler studier behövs göras för att verifiera resultatet. Man undersökte också om detektionen av Beta-galaktosidas kunde förbättras genom att tillsätta Bovin serumalbumin (BSA) för att inhibera resterande ozon i prover som annars skulle kunna inaktivera Beta-galaktosidas. Resultaten indikerade dock inte någon effekt. Som ett sista experiment mättes även endotoxiner som frigjordes vid behandling, och det visade sig att när bakterier behandlas med ozon frigörs lipopolysackarider (LPS) och peptidoglykaner, vilket också kan tyda på lysis.

Nyckelord

Bakteriemi, Ozon, Sepsis, Septisk shock, Antibiotika resistans, Lysis, Beta-galaktosidas, Sonikering, Ozonkoncentration, Bovint serum albumin (BSA), Endotoxin

Acknowledgements

This project for the Master thesis was done at the company Sangair between January 2023 and June 2023. I would first like to thank Sangair for allowing me the opportunity to do my thesis work at a company that wants to combat a severe disease, and allowing me to understand the efforts behind it. I would like to thank my external supervisor Johan Seijsing for guiding me throughout this project and helping me with the lab work. As well as my main supervisor at KTH, Anders Andersson, for his supervision. I would also like to thank Robert Wallin at SeqCure Immunology for helping me with doing part of the endotoxin detection tests towards the end of the project.

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1. Introduction

1.1 Project task and objectives

This master thesis project was done at the company Sangair, as part of the Master's degree in Medical Biotechnology at KTH Royal Institute of Technology, Stockholm. Sangair is developing a medical device to treat bacteremia - bacteria that can be found in the blood. The device intends to extract blood from the radial artery and treat the blood with ozone to kill the bacteria, before it is returned to the body.

The project aimed to develop a method to analyze lysis and to investigate if ozone had a lyzing effect on *E. coli*. Ozone exposure might disrupt the cellular membrane and cell wall. This could potentially release endotoxins and proteins into the blood, which can initiate an inflammatory response in patients, but is also an excellent way of killing bacteria. To measure bacterial lysis, *E. coli* was transformed with the pUC-19 plasmid for it to produce the enzyme Beta-galactosidase. After the bacteria had been treated with ozone, the amount of released Beta-galactosidase was analyzed using a chlorophenol red assay and the viability was evaluated. To further investigate the lysis, an endotoxin test to measure lipopolysaccharides (LPS) and lipoteichoic acid was done.

1.2 Background

Bacteremia is a pathological condition that that is defined as bacteria found in the bloodstream. Bacteremia can affect healthy individuals following a small medical procedure, or by a breach of the cutaneous barrier due to a wound. Hospitalized individuals and particularly those with peripheral venous catheters, are extra susceptible for bacteremia.[1,2] When the bacteria enter the body they will start to colonize *in situ* and the innate immune system is usually clearing the infection, leading to an asymptomatic course. However, if the immune system is overwhelmed or if it is impaired, the bacteria can evade the immune system and spread further in the circulatory system, culminating in a life-threatening complication, sepsis. [3]

Sepsis, defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection, is a a dangerous condition and if left untreated, poses a fatal threat to affected individuals.[4]

According to the World Health Organization (WHO), sepsis affects 49 million people worldwide, leading to 11 million fatalities annually.[7] Notably, around 42,6 % of the patients that get admitted to an intensive care unit (ICU) globally, are treated for sepsis, and 26,7 % of patients in hospitals generally, are treated for sepsis.[7] Sepsis is a disease that affects low-resource countries the most, where 85 % of the cases comes from sub-Saharan Africa, Oceania, and south-east Asia. However, it remains a significant health challenge also in high-resource countries.[8] In Europe it is estimated that 700,000 people die from sepsis every year.[7]

The most common symptoms that show when a person has bacteremia are fever, chills, and shivering. A person can also show more severe side effects such as quick breathing, rapid heartbeat, and changes in mental state.[5] When the pathogen finds its way into the host it is the innate immune system that will first react to it. This includes neutrophils, NK-cells, and macrophages, and these cells are not specific but are always active in the host. Once these bind to the pathogen they will activate cytokines like TNF-alpha, IL-1, and IL-6, and also caspases.[6] The cytokines will cause inflammation and activate the

adaptive immune system which is more specific towards the pathogen. During bacteremia, the immune system may spiral and cause more severe reactions which are described as sepsis. These reactions can lead to an immune response where the collateral damage is the death of tissues and cells in the organs.[6] What causes this is the reduced delivery of oxygen to the organs, due to hypoperfusion. Hypoperfusion is a result of the effect from the cytokines, TNFalpha and IL-1B that cause interference with the mitochondrial function of cardiac myocytes. Because of the oxygen reduction, it will result in anaerobic glycolysis and increased production of lactic acid. Because of the increased inflammatory state in the body, reactive oxygen species (ROS) will also cause a reduction in the function of mitochondria and ATP levels in cells, which can lead to organ failure.[6]

To treat bacteremia and sepsis, antibiotics are used. Antibiotics have been the cornerstone of antimicrobial therapy against bacterial infections since their discovery.[8] However, the growing antimicrobial resistance (AMR) poses a big threat to this class of molecules, and the WHO has declared this as one of the top 10 global public health threats.[9] The driver of resistance is multifactorial, including the misuse and overuse of antibiotics, suboptimal sanitation and hygiene for animals and humans, and inadequate awareness of the issue.[9] Antibiotics kill bacteria by inhibiting RNA synthesis, DNA synthesis, cell wall synthesis, and protein synthesis. Bacteria have evolved over millions of years and have genetic plasticity enabling them to adapt to environmental threats by evolving novel mechanisms of resistance. Two such mechanisms are mutational resistance and horizontal gene transfer, the latter facilitating acquisition of AMR genes from other bacteria through transformation, transduction or conjugation.[10] Bacteria can take up free DNA from its surrounding environment, which is called transformation. It can also transduct DNA from bacteria and virus, or actively transfer DNA between bacteria, which is called transduction and conjugation. Given the threat of AMR, new strategies, such as Sangairs'ozone mediated bacterial lysis, needs to be developed to kill bacteria.

Ozone, O_3, is an inorganic molecule, and is in nature generated from dioxygen (O_2), by the UV radiation in the earths' atmosphere and electrical discharges. It was discovered in 1839 and has since then found other applications in various industries, such as food, milk and drinking water for its bactericidal properties.[11] The mechanism by which ozone interact with bacteria, is inactivation of the cells through destruction of the bacterial cell membrane and cell wall, resulting in the release of intracellular compounds and leading to that the cells may lyse.[12] Upon interacting with the bacteria, ozone will decompose to O_2 and O. The single oxygen atom, a strong oxidizer, will have a microbicidal effect on the bacteria.[12]

1.2.1 Method development

A transformation with the pUC-19 plasmid was done in *E. coli* in order for it to express Beta-galactosidase. The double stranded DNA plasmid was devised in 1985 by Joachim Messing and contains several functional sites such as the Beta-galactosidase gene (*LacZ*), the multiple cloning site (MCS), an origin of replication site (ORI) and a gene for ampicillin resistance (amp) encoded by the β-lactamase gene (Figure 2). The ampicillin resistance gene enabled transformed bacteria to grow on an ampicillin-containing plate, confirming successful transformation. [13]

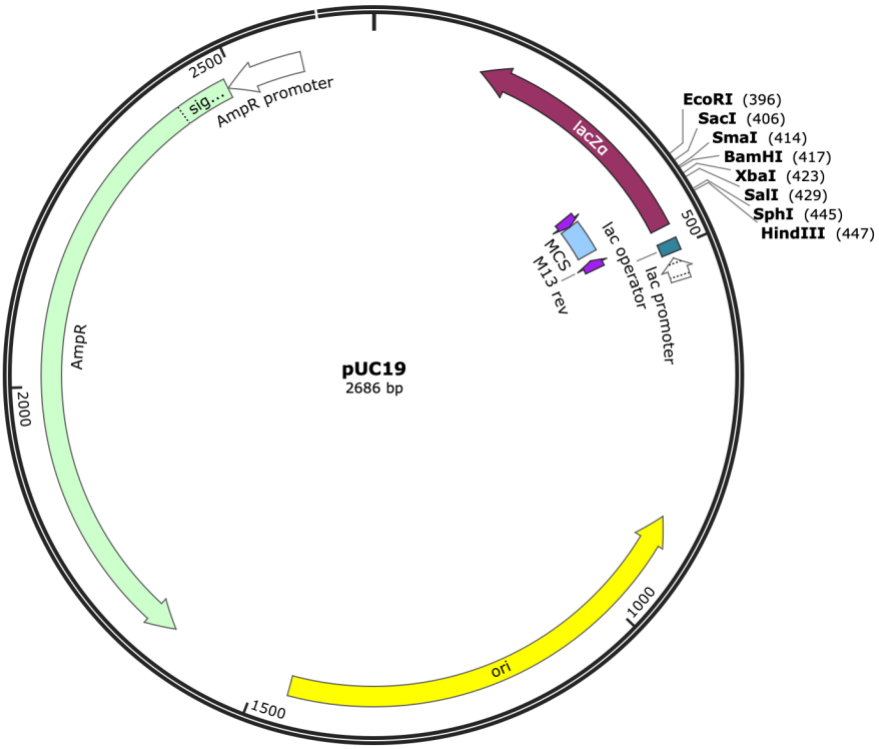

Figure 2: Image showing the plasmid pUC19 containing the Beta-galactosidase gene, LacZα. (This image was created using SnapGene)

To insert the plasmid, a bacterial transformation was performed. Transformation was done using a heat shock method, invented in 1928 by Fred Griffith.[14,15] When using the heat shock method, cells are exposed to increasing temperature, going from 0 to 42 °C. The environmental stress alters the membrane of the bacteria. These alterations result in the formation of fusion sites between the cell membrane and the cell wall, enabling uptake of foreign materials of sufficiently small size such as a plasmid. IPTG was then added, that acts as a lac repressor inhibitor, to be able to induce the Lac Z synthesis and produce Beta-galactosidase. To be able to measure the Beta-galactosidase presence and enzymatic activity, Chlorophenol red-β-D-galactopyranoside (CPRG) was added to the lysed cells. Beta-galactosidase catalyzes the hydrolysis of the CPRG, resulting in the yellow CPRG being converted into chlorophenol red, whereby the solution turns red (Figure 3). The absorbance of chlorophenol red was measured using a spectrophotometer at a wavelength at 570 nm.[16]

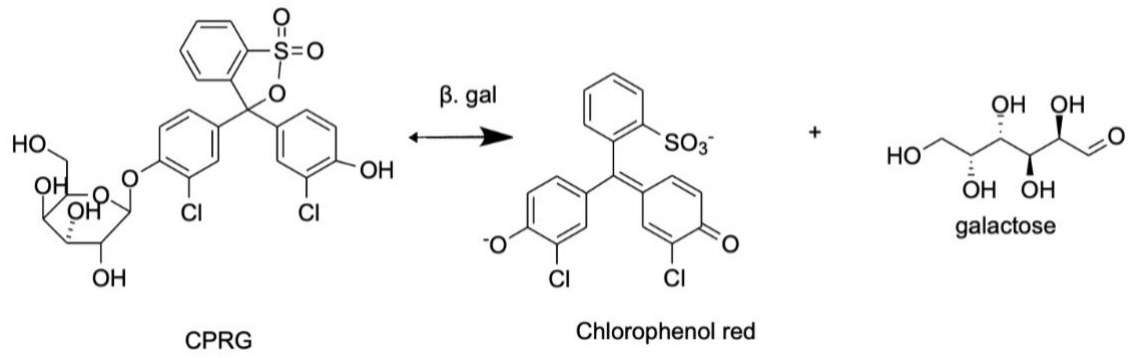

Figure 3: The chemical reaction of CPRG catalyzed by Beta-galactosidase. Beta-galactosidase will cleave CPRG substrate into chlorophenol red and galactose products. (Image created using ChemDraw)

1.2.2 Ozone treatment

The treatment step with ozone was done in an ozone rig. (Figure 4).

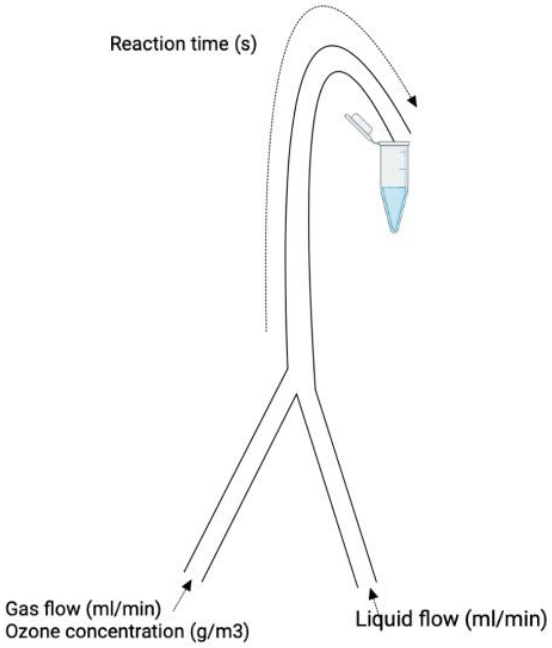

Figure 4: A simplified illustration of the setup in the ozone rig. Oxygen gas goes from the oxygen tank to the ozone generator (g/m3). The gas goes through the mass flow controller (gas flow) (ml/min) before being mixed with the liquid. The liquid from a bacterial suspension will go through a pump where the liquid flow can be altered (ml/min). The bacterial suspension then goes into the reaction chamber where it will meet the ozone gas before being collected at the output. (This image was made using BioRender)

Three parameters were varied in the ozone rig during the experiments. The ozone concentration, the gas flow, and the liquid flow. Increase in ozone concentration, keeping the two other parameters constant, leads to more ozone molecules that can react with the bacteria in the suspension. Increase in gas flow, keeping the other two parameters constant, lead to more ozone gas going into the bacterial suspension. And finally, increase in liquid flow, keeping the other two parameters constant, leads to less interaction time with the ozone. After treatment in the ozone rigg the treated bacterial suspension was added to cystine lactose electrolyte deficient (CLED)-plates to measure the viability (colony formin units (CFU)/ml). The number of viable *E. coli* could be observed on the plate as yellow colonies. CLED plates contains lactose and bromothymol blue. When *E. coli* ferments the lactose, the pH is lowered, and the medium appears yellow.[17] The bacterial suspension was also centrifuged to remove cell debris, and then the supernatant was added to a 96-well plate with CPRG, to measure Beta-galactosidase activity with a plate reader at 570 nm.

1.2.3 Endotoxin detection

As a final experiment, an endotoxin measurement was conducted to measure the amounts of cell wall components in the treated samples.

Endotoxins are components in the cell wall of *E. coli* bacteria. They have the capacity to trigger the innate immune system which can result in fever, septic shock and death. Recognition of endotoxins are vital for bacterial detection by the immune system. However, the release of endotoxins through

cellular lysis may trigger an immune reaction that is even stronger than the live bacteria themselves.[18] Therefore, it is important to test for endotoxins as these may be released during treatment with ozone.

An endotoxin detection kit from Invivogen was used to measure endotoxin in treated samples (FIGURE 5). Cells expressing Toll-like receptor 2 (TLR2) bind to lipoproteins and peptidoglycans.[20] The TLR2 triggers NF-kB that induces the production of secreted alkaline phosphatase (SEAP). QUANTI-Blue substrate was then added, creating a purple/blue colored product when hydrolyzed by SEAP. The number of endotoxins was determined by measuring the absorbance in a spectrophotometer at 625 nm.

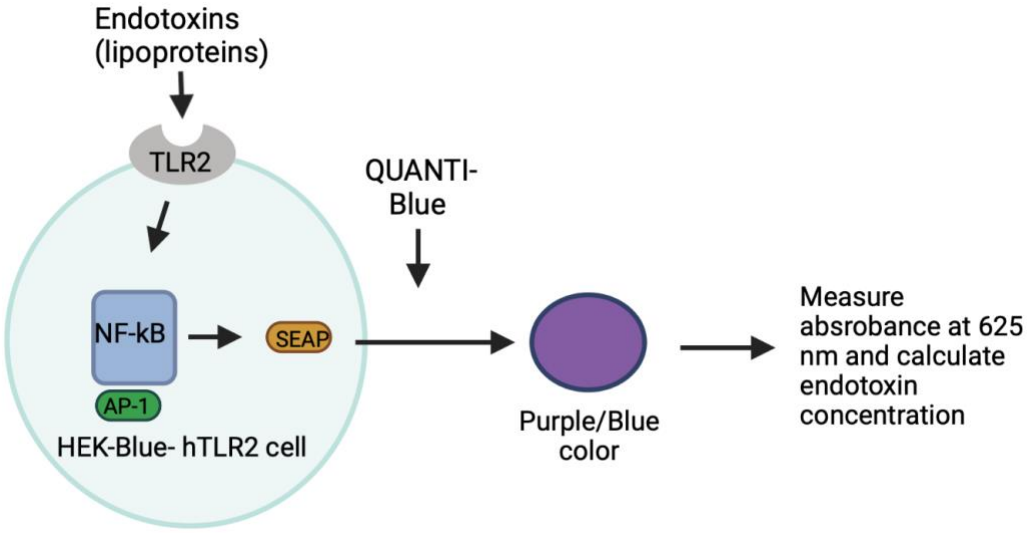

Figure 5: Mechanism of action of the endotoxin detection kit from Invivogen. (This image was generated using BioRender)

1.3 Project workflow

The workflow of the project can be seen in Figure 1.

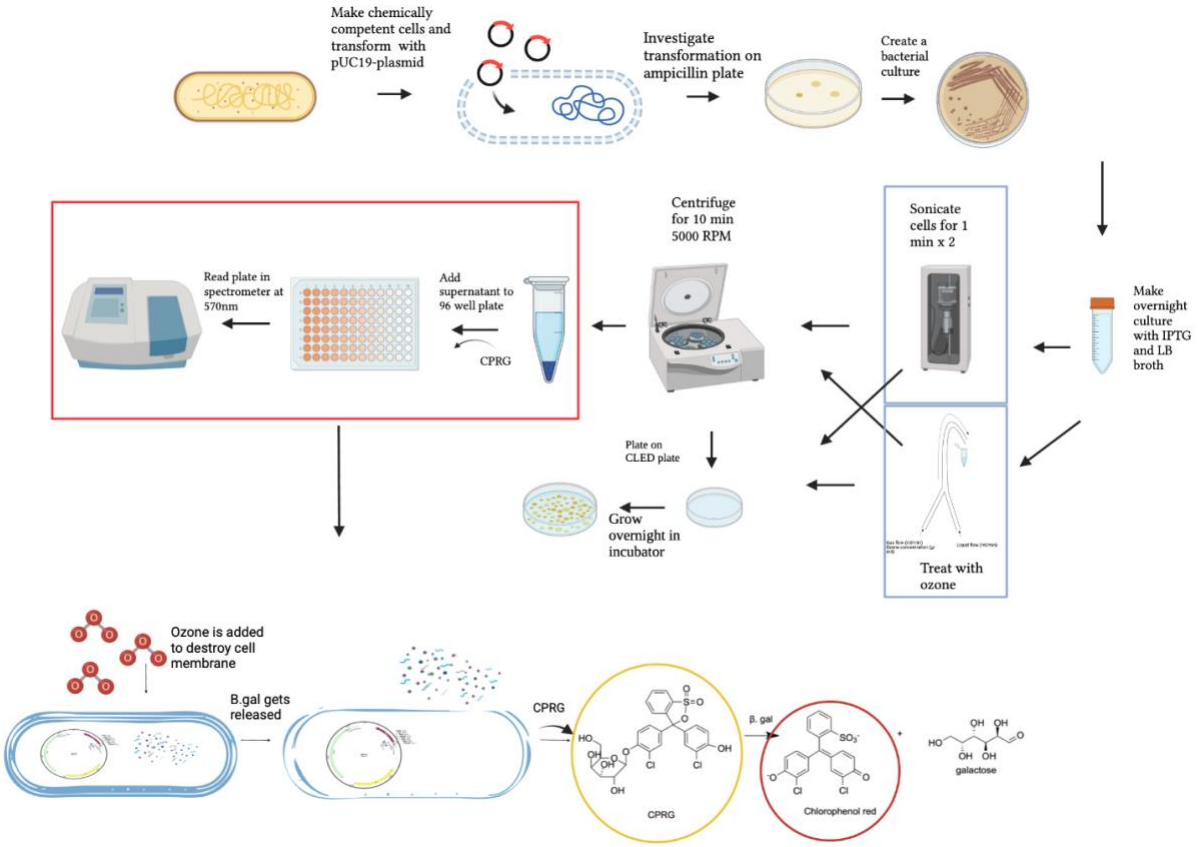

Figure 1: The goal of the project is to find out if bacteria lyse upon treatment with ozone. To investigate this we treated pUC-19 transformed BL21 *E. coli*, with ozone or sonication. The treatments lead to intracellular leakage of Beta-galactosidase enzyme. The enzymatic substrate chlorophenol red-β-D-galactopyranoside (CPRG) was then added to react with the released Beta-galactosidase to produce the enzymatic product chlorophenol red that could be measured in a plate reader. An in-depth view of the reaction that gives the absorbance can be seen under the red square.

2.Material and methods

2.1 Bacterial Strains

Two different bacterial strains were used; BL21 and DH5α. BL21 contains a functional gene encoding Beta-galactosidase in its genome but it was still transformed to produce more Beta-galactosidase.

2.2 Making *E. coli* competent and bacterial transformation

To make the cells competent, the protocol by Hanahan, D. et al was followed.[21] Bacteria was cultured overnight in an incubator at 37 °C and shaken at 200 RPM. The following day the culture was subcultured by adding 1 ml of the overnight culture to 99 ml LB (Substratenheten, Karolinska Sjukhuset, Stockholm, Sweden) and incubated at 37 °C at 200 RPM for 4 hours. First, it was centrifuged at 4000 RPM for 10 minutes discarding the supernatant. It was then washed with the 0,1 M $CaCl_2$ buffer (Sigma-Aldrich, Saint Louis, USA), followed by centrifuging it for 10 minutes at 4000 RPM. Lastly, the resulting cell pellet was stored by adding 0,1 $CaCl_2$ + 15% glycerol buffer (Sigma-Aldrich, Saint Louis, USA).

For transformation of the BL21 and DH5a strains with the pUC-19 plasmid, the protocol from Bergmans, H. et al was followed.[14] The steps for this included heat shock transformation, plating cells, followed by incubation. For the heat shock, 5 µl of the plasmid was added to thawed bacterial cells and was then incubated on ice for 30 minutes. The cells were then heat shocked by placing in a water bath at 42 °C for 30 seconds followed by 2 minutes on ice. 1 ml of LB (Substratenheten, Karolinska Sjukhuset, Stockholm, Sweden) was then added before incubating the cells at 37 °C at 200 RPM for one hour. 100 µl of the bacterial suspension was then added on an ampicillin plate. The following day he plate was checked for transformed colonies, and these were cultivated in liquid LB and frozen down as glycerol stocks in a -80°C freezer.

2.3 Determining Beta-galactosidase production in transformed bacteria

To produce Beta-galactosidase 0,1 mM IPTG (ThermoFisher, Waltham, USA) was added overnight to cultures of the BL21 and DH5a strains. 2 x 500 µl of each culture was added to four Eppendorf tubes. 5 µl of the 0,1 mM IPTG (ThermoFisher, Waltham, USA) buffer was added to one of the Eppendorf tubes of each strain, and as a control 5 µl of PBS (Substratenheten, Karolinska Sjukhuset, Stockholm, Sweden) was added to the other two Eppendorf tubes. After the tubes had incubated in a shaking incubator overnight, the bacteria were pelleted by centrifugation at 5000 RPM for 5 minutes, washed once and redissolved in 1xPBS (Substratenheten, Karolinska Sjukhuset, Stockholm, Sweden). The bacteria were then sonicated on ice for 2x1 minutes and centrifuged 5 minutes at 5000 RPM. 100 µl of each supernatant was added to 6 wells on a 96 well plate, and 100, 50, 25, 12,5, 6,25 µl of 5 mM CPRG buffer was added to the wells.

2.4 Determination of bacterial concentration for adequate signal

An overnight culture with DH5α, DH5α-pUC19, DH5α-pUC19 induced with IPTG, BL21-pUC19 and BL21-pUC19 induced with IPTG was made. The following day 1,5 $*10^8$ CFU/ml of each culture was added to 1 ml of PBS (Substratenheten, Karolinska Sjukhuset, Stockholm, Sweden), using a nephelometer (DensiChek, Marcy-l'Etoile France). 500µl from each culture was added to an

Eppendorf tube and was then sonicated for 2x1 min and centrifuged 5 minutes at 5000 RPM. The non-sonicated and sonicated samples were added to a 96-well plate. 110 µl of each culture was added to the top row with a concentration of 1.5 $*10^8$. Samples were then diluted 1:10 to create a dilution series. After this, 50 µl of 0,5 mM CPRG was added to each well and a plate reader (EZ Read 400, Biochrom, Cambridge, UK) was used to measure the absorbance of chlorophenol red at 570 nm.

The cultures were also plated on CLED plates with a bacteria concentration of 0,75 $*10^3$ CFU/ml and 0,75* 10^2 CFU/ml. 100 µl of the sonicated and non-sonicated DH5α, DH5α-pUC19, DH5α-pUC19 induced with IPTG, BL21-pUC19 and BL21-pUC19 induced with IPTG were added to the plates. An additional plate with only 100 µl of PBS (Substratenheten, Karolinska Sjukhuset, Stockholm, Sweden) was performed as a control.

2.5 Treatment with Ozone

2.5.1 Preparation of culture prior to treatment

The strain of BL21-pUC19 was sustained on an agar plate in a 4 °C fridge for the duration of the project. A single colony from the plate was inoculated into 50 ml of LB (Substratenheten, Karolinska Sjukhuset, Stockholm, Sweden) containing 50 µl 0,1 mM IPTG (ThermoFisher, Waltham, USA) and incubated at 37 °C, overnight. Ca 5 ml of the overnight culture was then added to a 50 ml solution with 50 ml 10x PBS (Substratenheten, Karolinska Sjukhuset, Stockholm, Sweden) and 445 ml deionized water to create a bacterial concentration of 1,5 $*10^7$ CFU/ml, using a nephelometer (DensiChek, Marcy-l'Etoile France).

2.5.2 Finding the right treatment time with ozone

The bacterial suspension was treated with ozone for 0s, 15 s, 30 s, 1 min, 2 min, 4 min, and 8 min at a fixed concentration of ozone, 20 g/m^3, a liquid flow of 50 ml/min, and the gas flow at 10 ml/min. At each timepoint, 300 µl of the suspension was extracted and transferred to an Eppendorf tube. This experimental procedure was executed in triplicates.
Following the treatment, each sample was divided into two, where one sample contained 200 µl, that was used for an enzymatic assay, and the other sample had 200 µl that was used to make dilutions for a viability assay. The first aliquot of each sample was centrifuged, and the supernatant was saved. Subsequently, the supernatant of each sample was pipetted to a 96 well plate at a concentration of 1,5$*10^7$ CFU/ml and diluted to a concentration of 1,5$*10^4$ CFU/ml. In each well, 50 µl of 0,5 mM CPRG was added, and an absorbance measurement of chlorophenol red was made using a plate reader at 570 nm (EZ Read 400, Biochrom, Cambridge, UK).
Furthermore, the latter aliquot of each sample was used to determine bacteria viability (CFU). Dilutions of 1,5$*10^4$ and 1,5$*10^3$ CFU/ml were made from each sample and added to CLED plates (Substratenheten, Karolinska Sjukhuset, Stockholm, Sweden). The following day the CFU was calculated.

2.5.3 Investigating if Beta-galactosidase becomes inactivated by ozone

The bacterial suspension was sonicated 2x1 min on ice, centrifuged to pellet the bacteria, and the supernatant was saved. 200 µl of the supernatant was added to 5 ml of 1x PBS (Substratenheten, Karolinska Sjukhuset, Stockholm, Sweden) and then aliquoted into 5 falcon tubes. Four of the tubes were then treated with ozone for one minute each by adding ozone into the falcon tube with the ozone

concentration at 12,7 g/m^3 and the gas flow was adjusted from 5, 10, 15 and 20 ml/min for each of the four samples. Once the samples were treated, 100 µl of each sample from the different time points, and the untreated sample, were pipetted into a 96 well plate. The different samples were diluted from 100% to 0,1 % in steps of 10x. 50 µl of CPRG was then added to each well and a plate reader (EZ Read 400, Biochrom, Cambridge, UK) was used to measure the absorbance at 570 nm .

2.5.4 Treatment in ozone rigg with different ozone concentration

The bacterial suspension was treated with ozone in the ozone rigg, with the ozone concentration being 6,9, 7,5, 10, 15 and 20 g/m^3, the liquid flow was set to 50 ml/min and the gas flow was set to 5 ml/min. A sample was taken after each change of ozone concentration. After this, the same steps as described under 2.5.2, following treatment and measuring absorbance and viability, was done.

2.5.5 Treatment in ozone rigg with different liquid flow rates

The bacterial suspension was treated with ozone with the ozone concentration being 6,0 g/m^3, the gas flow was set to 5,1 ml/min and the liquid flow was changed from 25, 50, 100, 200, and 300 ml/min. A sample was taken after each change of flow rate. After this, the same steps as described under 2.5.2, following treatment and measuring absorbance and viability, was done.

2.5.6 Treatment in ozone rigg with Beta-galactosidase and different concentrations of Bovine Serum Albumin

The bacterial suspension was sonicated to release the Beta-galactosidase for 2x1 min. The suspension was centrifuged at 5000 RPM x 5 min and he supernatant aliquoted equally into seven 50 ml falcon tubes. To six of the tubes 3.125, 6.25, 12.5, 25, 50 and 100 µl of 10% bovine serum albumin (BSA, Saveen & Werner, Limhamn, Sweden) was added. No BSA was added to the seventh control tube. All samples were treated in the ozone rigg, except for the control that was untreated. The settings in the rigg were the following: ozone concentration 6,0 g/m^3, gas flow 5 ml/min and the liquid flow 50 ml/min. After the treatment of each sample, they were pipetted to a 96 well plate, were a dilution of the samples to 1,5*10^5 CFU/ml times were made. 50 µl of CPRG was then added to each well before measuring absorbance in a plate reader at 570 nm (EZ Read 400, Biochrom, Cambridge, UK).

2.5.7 Treatment of *E. coli* in ozone rigg with different liquid flows and addition of Bovine Serum Albumin

In five Eppendorf tubes 100 µl of BSA (Saveen & Werner, Limhamn, Sweden) was added. The bacterial suspension was then treated in the ozone rigg with different liquid flows comprising of 12,5 ml/min, 25 ml/min, 50 ml/min, and 100 ml/min. The gas flow was 5.0 ml/min and the ozone concentration was 6 g/m^3. A sample was added to an Eppendorf tube without BSA as a control before treatment in the ozone rigg. 0,9 ml of the treated suspension was then added as a control to one of the Eppendorf tubes with BSA directly after treatment. After this, the same steps as described under 2.5.2, following treatment and measuring absorbance and viability, was done.

2.5.8 Measurement of endotoxins after treatment with ozone, with different liquid flows and addition of Bovine Serum Albumin

In 18 Eppendorf tubes, 100 µl of BSA (Saveen & Werner, Limhamn, Sweden) was added. The setup in the ozone rigg was 5,0 ml/min gas flow, an ozone concentration of 6 g/m^3 and with four different

liquid flows comprising of 12,5 ml/min, 25 ml/min, 50 ml/min and 100 ml/min. Before the bacterial treatment started, a negative control was run in the ozone rigg with the ozone turned off. A control sample was run in the set up with ozone, but without BSA in the Eppendorf tube. After this, the bacterial suspension was run in the setup where three aliquots á 0,9 ml of the treated suspension was added to one of the Eppendorf tubes with BSA directly after each change of the liquid flow.

The samples were then aliquoted into two Eppendorf tubes á 500 µl each. One of the aliquots was centrifuged and the supernatant of each sample was saved. 100 µl of the supernatant of each sample was added to a 96 well plate at the concentration of $1,5*10^7$ CFU/ml, and then 1:10 dilutions were done to a concentration of 10^4 CFU/m. In each well, 50 µl of 0,5 mM CPRG was added, and absorbance measurements were done using a plate reader at 570 nm (EZ Read 400, Biochrom, Cambridge, UK). The other aliquot was used to measure viability (CFU). This was achieved by making 1:10 dilutions to $1,5*10^3$ CFU/m from each sample, which were then added to CLED plates (Substratenheten, Karolinska Sjukhuset, Stockholm, Sweden). The following day the number of CFU was determined through counting.

The remaining supernatant from the absorbance aliquot was used for endotoxin detection. The protocol from Invivogen was followed and the samples were prepared in triplicates.[18] In short, the first day the sample dilutions and HEK-Blue Endotoxin standard dilutions were prepared and 20 µl was added on separate rows in a 96 well. 20 µl endotoxin-free water was then added to both dilutions. 20 µl 0,1 EU/ml HEK-Blue Endotoxin standard spike solution was then added to one row of the sample dilutions. An HEK-Blue 4 cell solution was then prepared.160 µl of the HEK-Blue 4 cell solution was then added to all the wells and the plate was then stored in a CO2 incubator at 37 Cº for 18 hours for the cells to settle. On the second day QUANTI-Blue was prepared and warmed to 37 Cº for 30 minutes. 20 µl of each supernatant from the plate was then added to a new plate were 180 µl QUANTI-Blue was added and incubated for 2 hours at 37 Cº. The absorbance was then read at 655 nm.

3. Results

3.1 Bacteria, transformation, Beta-galactosidase expression and substrate detection

The first part involved finding a suitable amount of CPRG substrate to utilize in the experiments. *E. coli* strains BL21 and DH5a were transformed with a Beta-galactosidase expressing plasmid pUC19. Expression of the gene was confirmed by adding different concentrations of CPRG substrate to different amounts of sonicated induced cells (Figure 6). 50 µl of CPRG at a concentration of 0,05 mM was found to be a concentration suitable for the following experiments giving values within the dynamic range of the plate reader after overnight incubation.

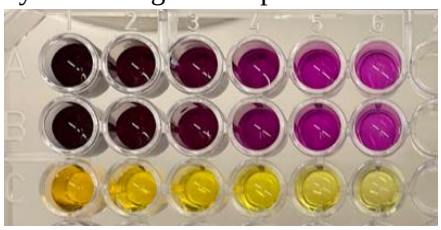

Figure 6: Optimizing of CPRG concentration. In row 1, 100 µl of BL21-pUC19-IPTG in each well A1-A6, in row 2, 100 µl BL21-pUC19 in each well B1-B6, and in row 3 100 µl DH5a in each well, C1-C6. In column 1A-C, 100 µl of CPRG buffer, 2A-C, 50 µl of CPRG buffer, 3A-C, 25 µl of CPRG buffer, 4A-C, 12,5 µl of CPRG buffer, 5A-C, 6,25 µl of CPRG buffer, 6A-C, 3,125 µl of CPRG buffer was added.

3.1.1 Determining what bacterial strain to use for the experiments with ozone

The appropriate bacterial quantity and strain to be utilized in the experiments was then determined. The viability was reduced significantly in all samples subjected to sonication, indicating cell death and potentially lysis (Figure 7). For the Beta-galactosidase activity it was the sonicated samples that gave much stronger signals of chlorophenol red product compared to non sonicated samples of the same strains, likely indicating bacterial lysis (Figure 8). The signal was strongest in the sonicated BL21-pUC19 induced with IPTG, and at a bacterial concentration of $1{,}5*10^8$ CFU/ml. But, since the absorbance was outside of the measuring range, a bacterial concentration of $1{,}5*10^7$ was chosen to use for the following experiments (Figure 8). Notably, the BL21 strain already possessed a gene for Beta-galactosidase, but it was still transformed with the plasmid to be able to produce more Beta-galactosidase. Since it was observed that the absorbance of chlorophenol red product was higher with the addition of IPTG for BL21-pUC9, it was used in the following bacterial colony experiments.

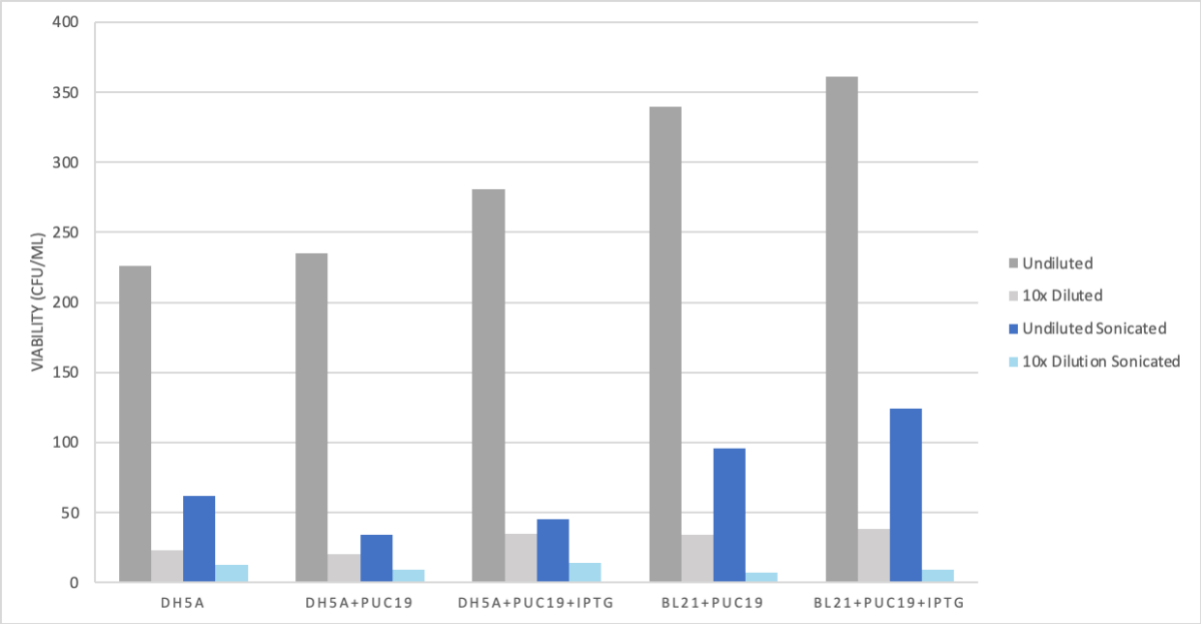

Figure 7: Measurement of viable bacteria after sonication or no sonication. Viable bacteria of DH5α, DH5α-pUC19, DH5α-pUC19 induced with IPTG, BL21-pUC19, BL21-pUC19 induced with IPTG after treatment with sonication or untreated. Both non-diluted ($1{,}5*10^8$ CFU/ml) and 10x diluted($1{,}5*10^7$ CFU/ml) samples can be seen.

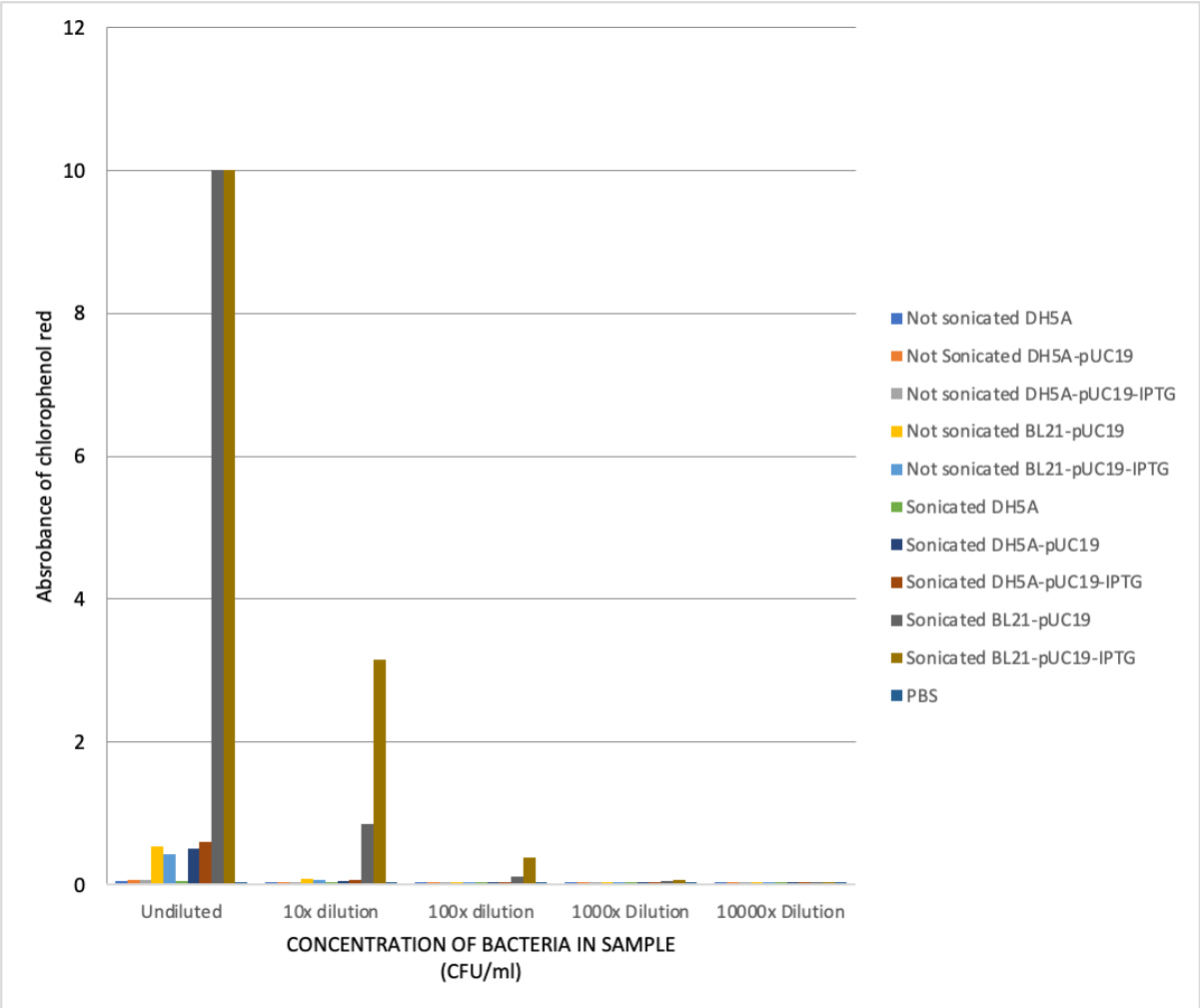

Figure 8: Measurement of Beta-galactosidase activity after sonication or no sonication. Absorbance of chlorophenol red product in sonicated samples and untreated samples of of DH5α, DH5α-pUC19, DH5α-pUC19 induced with IPTG, BL21-pUC19, BL21-pUC19 induced with IPTG, and PBS. Cell dilutions from $1,5*10^8$ to $1,5*10^4$ CFU/ml was done.

3.2 Detecting lysis of bacteria after treatment with ozone

3.2.1 Determining treatment time with ozone

To be able to see if ozone had a lysing effect on the bacteria, settings, such as treatment time, needed to be optimized. In the first trial to detect lysis following treatment with ozone, an exploration of optimal treatment time was conducted by bubbling ozone in a falcon tube. It was found that 15 seconds of treatment time was sufficient to lose almost all cell viability, while the absorbance of chlorophenol red at 570 nm was approximately 0,05, which was equivalent to the background signal (i.e. the absorbance obtained after much longer time) (Figure 9). From this result it shows that the diluted sample contained more Beta-galactosidase than the undiluted sample. This could indicate that ozone might still be found in the undiluted sample, and inactivating the Beta-galactosidase. When the dilutions are made, less ozone will then follow to the diluted sample and making it harder to inactivate the Beta-galactosidase. Also, if the cells had lysed, given that no viability can be seen (Figure 9), higher concentrations of chlorophenol red should also have been seen which also gives more proof for this hypothesis.

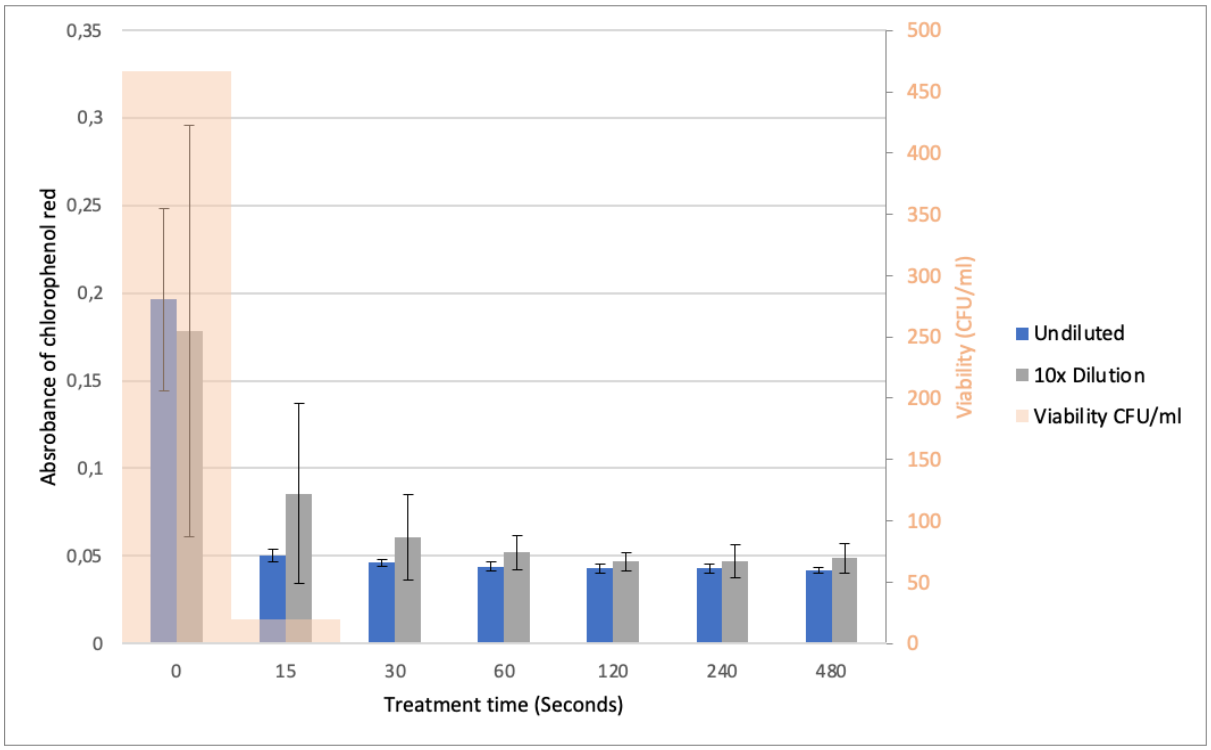

Figure 9: Measurement of Beta-galactosidase activity and viability after different treatment times with ozone. Absorbance of chlorophenol red after treating IPTG-induced BL21-pUC-19, induced with IPTG, with ozone (20 g/m^3) for different treatment times. Both the undiluted ($1.5*10^8$ CFU/ml) and 10x diluted($1,5*10^7$ CFU/ml) for the sample can be seen. The viability after each time point is also shown.

3.2.2 Investigating if Beta-galactosidase is inactivated by ozone, and finding the optimal ozone concentration for treatment

Since the absorbance of chlorophenol red product was noticeably low in the previous experiment even though the cell viability was low (Figure 9), it was then hypothesized that ozone had an inactivating effect on the potentially released Beta-galactosidase. An experiment with treatment of ozone on Beta-galactosidase protein in cell lysates in PBS solution was therefore conducted with different gas flows. No absorbance could be measured following treatment, in contrast to the untreated sample (Figure 10). Since no absorbance could be seen, it was determined that for the following experiments, the gas flow should be kept as low as possible to be able to measure any possible activity of Beta-galactosidase, and 5 ml/min was set for the following experiments.

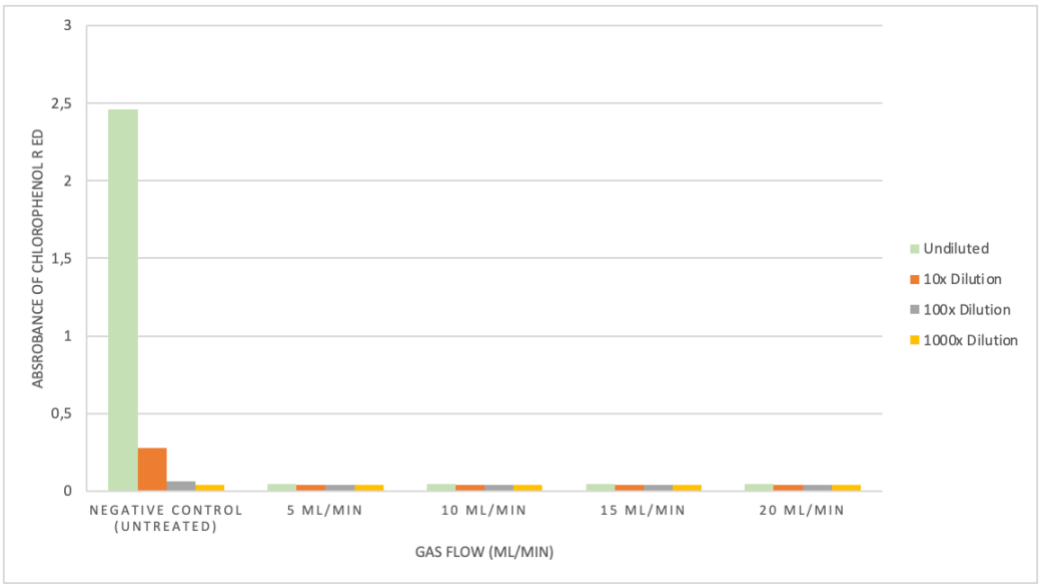

Figure 10: Measurement of Beta-galactosidase activity in PBS with different ozone gas flows. Absorbance of chlorophenol red at 570 nm after Beta-galactosidase in a PBS solution was treated with four different gas flows, with four different dilutions from the lysate of the concentrations of the bacteria; undiluted ($1,5*10^7$ CFU/ml), 10x diluted ($1,5*10^6$ CFU/ml), 100x dilution ($1,5*10^5$ CFU/ml), 1000x dilution($1,5*10^5$ CFU/ml).

To then find the optimal ozone concentration for bacterial lysis, while preserving Beta-galactosidase activity, an investigation was conducted with different ozone concentrations while running the experiment in the ozone rigg instead of a falcon tube, to achieve a shorter treatment time. The results indicate that bacterial viability was still detectable at an ozone concentration of 6,9 g/m^3. A chlorophenol red product absorbance was also observed. Because of these findings, the following experiments were centered around this ozone concentration and done in the ozone rig and not in falcon tubes (Figure 11). Even though the step in ozone concentration from 6,9 g/m3 to 7,5 g/m3 is not that high, no viability or Beta-galactosidase activity could be measured at 7,5 g/m3. This could possibly be because of the instability of the ozone generator, as it was found to sometimes show a higher concentration of ozone during the experiments, than the one that was initially set. This could therefore have affected the result.

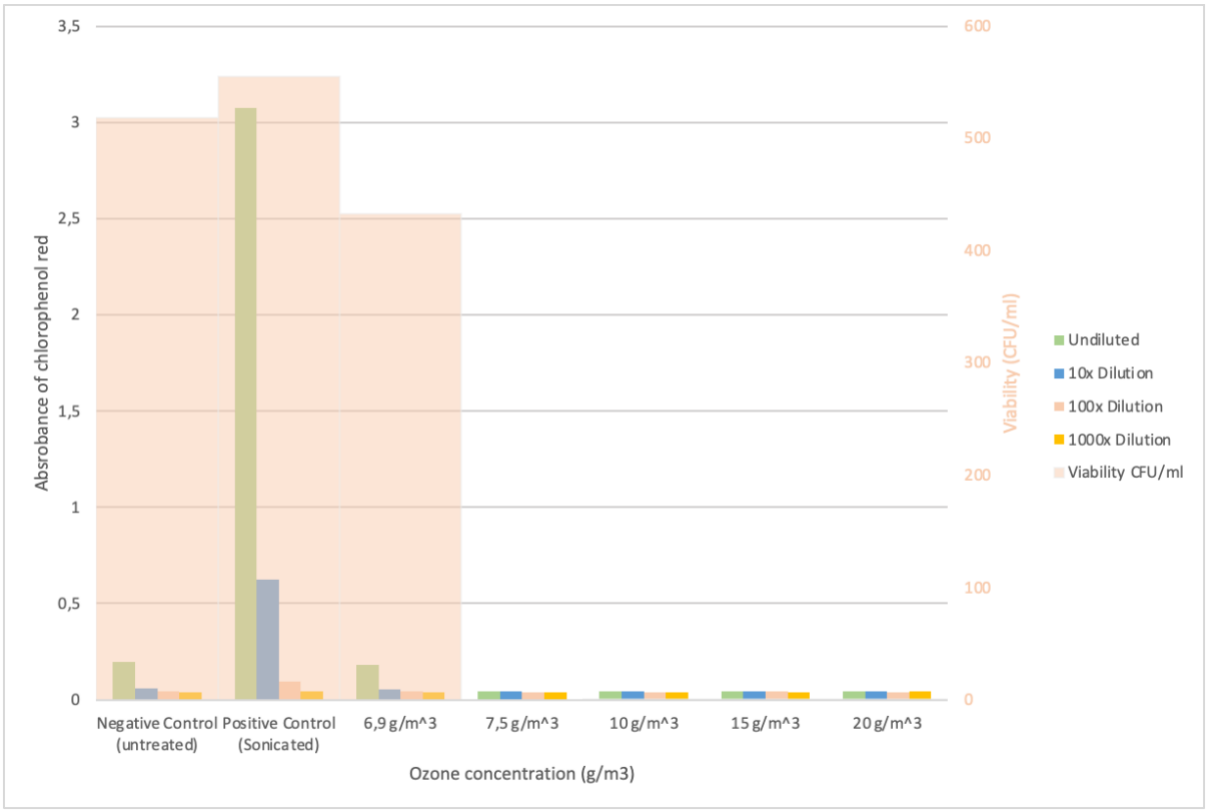

Figure 11: Measurement of Beta-galactosidase activity and viability after treatment with different ozone concentrations. Absorbance of chlorophenol red at 570 nm after BL21-pUC19, induced with IPTG, was treated with five different ozone concentrations in g/m^3, in four different dilutions of the treated sample undiluted ($1{,}5*10^7$ CFU/ml), 10x diluted ($1{,}5*10^6$ CFU/ml), 100x dilution ($1{,}5*10^5$ CFU/ml), 1000x dilution($1{,}5*10^4$CFU/ml). The viability after each change of concentration is also shown.

3.2.3 Investigating if less contact with ozone affects viability and Beta-galactosidase activity

Once the optimal ozone concentration and gas flow had been established, the last parameter that was subject to alteration was the liquid flow. At a liquid flow rate of approximately 25 ml/min, half of the bacteria were dead, and an absorbance of chlorophenol red was still detectable (Figure 12). These results indicate that the most effective configuration was with an ozone concentration of 6 g/m^3, a liquid flow rate of 25 ml/min, and a gas flow at 5 ml/min to kill half bacteria while still being able to detect Beta-galactosidase.

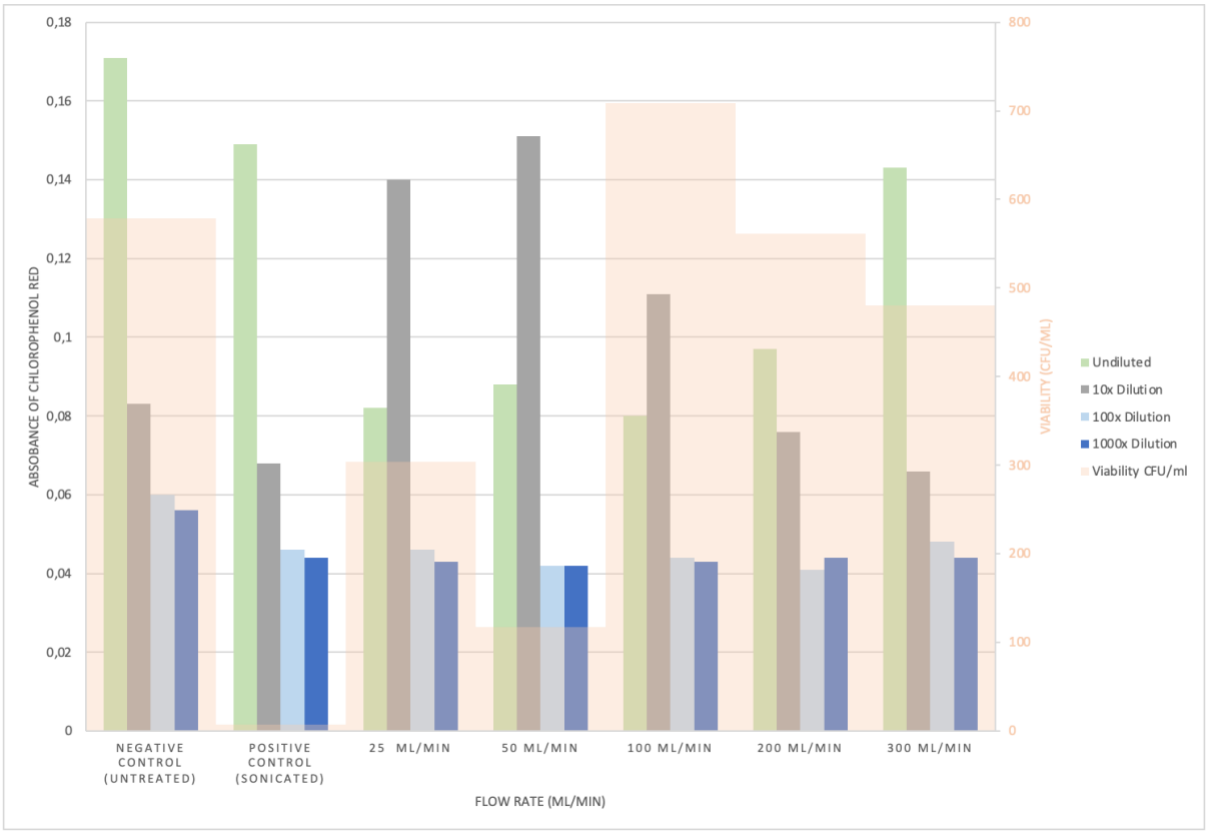

Figure 12: Measurement of Beta-galactosidase activity and viability after treatment with ozone at different liquid flows. Absorbance of chlorophenol red at 570 nm after BL21-pUC19, induced with IPTG, was treated with ozone at five different flow rates, in four dilutions of the treated sample undiluted ($1,5*10^7$ CFU/ml), 10x diluted ($1,5*10^6$ CFU/ml), 100x dilution $1,5*(1,5*10^5$ CFU/ml), 1000x dilution($1,5*10^5$ CFU/ml).. The viability after each change in flow rate is also shown.

3.3 Investigating Beta-galactosidase detection, after treatment with ozone, with added Bovine Serum Albumin

An additional factor, bovine serum albumin (BSA) was added to see if the Beta-galactosidase detection post ozone treatment could be improved. Since we earlier observed that ozone had an inactivating effect on Beta-galactosidase this could indicate that some of the released Beta-galactosidase may be inactivated by the ozone and the result could therefore be misleading (Figure 10). To see if more Beta-galactosidase could be measured, BSA was added to the samples, which mimics human albumin found in blood. The intention was to utilize ozone to lyse the bacteria, with BSA serving to neutralize any excess ozone that might inactivate Beta-galactosidase. The first experiment was done with only Beta-galactosidase protein in lysate from bacteria and PBS in the sample, with addition of BSA directly after the treated liquid was collected. The results from this experiment indicate an optimal concentration range to detect chlorophenol red was between 75,26 nM and up. (Figure 13). The reason for such strong absorbance of the negative control that is untreated, could be that the Beta-galactosidase is preserved very well in the PBS solution together with the BSA.

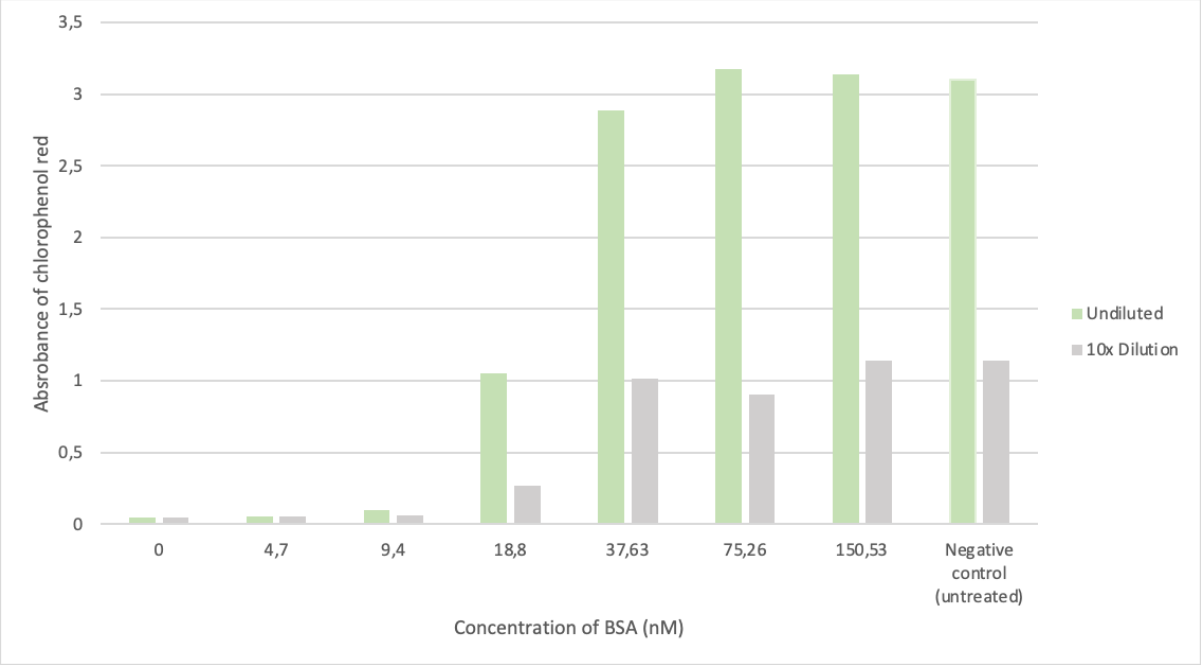

Figure 13: Measurement of Beta-galactosidase activity, with BSA, treated with ozone Absorbance of chlorophenol red at 570 nm after treatment in ozone rigg, with only PBS and Beta-galactosidase in the sample, after BSA was added at different concentrations. The lysate of lysed bacterial suspension at that given concentration was used, undiluted ($1,5*10^7$ CFU/ml) and 10x diluted ($1,5*10^6$ CFU/ml). The negative control contained Beta-galactosidase, BSA and PBS.

In the next step an experiment with different flow rates was done with whole bacterial cells, and with addition of BSA directly into the Eppendorf tubes when samples were collected post treatment. This was done to quench any additional ozone left in the tube. The viability decreased for the treated samples with a lower liquid flow, and increased for the samples at a higher liquid flow. As for the activity of Beta-galactosidase, no indication of an increase of absorbance could be seen when BSA was added. The treated sample without BSA at 12,5 m/min showed a higher absorbance of Beta-galactosidase than the samples with BSA (Figure 14).

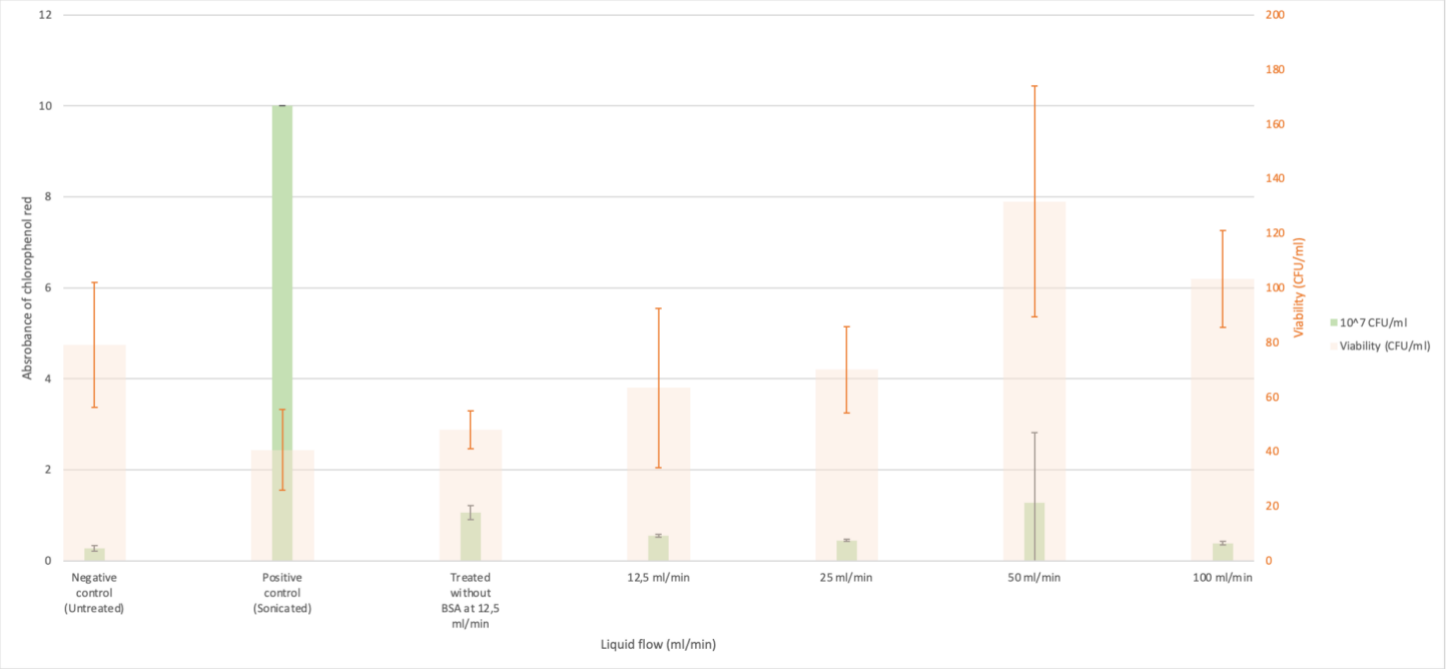

Figure 14: Measurement of Beta-galactosidase activity after treatment with ozone and addition of BSA. Absorbance of chlorophenol red at 570 nm and viability can be seen after treatment in the ozone rigg with different liquid flows, with addition of BSA after each treatment step.

3.4 Measuring endotoxins after treatment with ozone

After treatment in the ozone rigg, the supernatant was analyzed for released endotoxins. When using TLR2 cells for detection, the amount of released endotoxins appear higher for the treated samples with BSA, compared to the negative control and the sample treated without BSA. The only exception is the samples at 50 ml/min which almost had the same absorbance as the negative control (Figure 15). The treated sample without BSA shows a very low amount of absorbance even though the viability, as seen in Figure 14, is very low and endotoxins should have been released. This could be due to that the additional ozone that is left in the sample has reacted with the endotoxins and inactivated them.

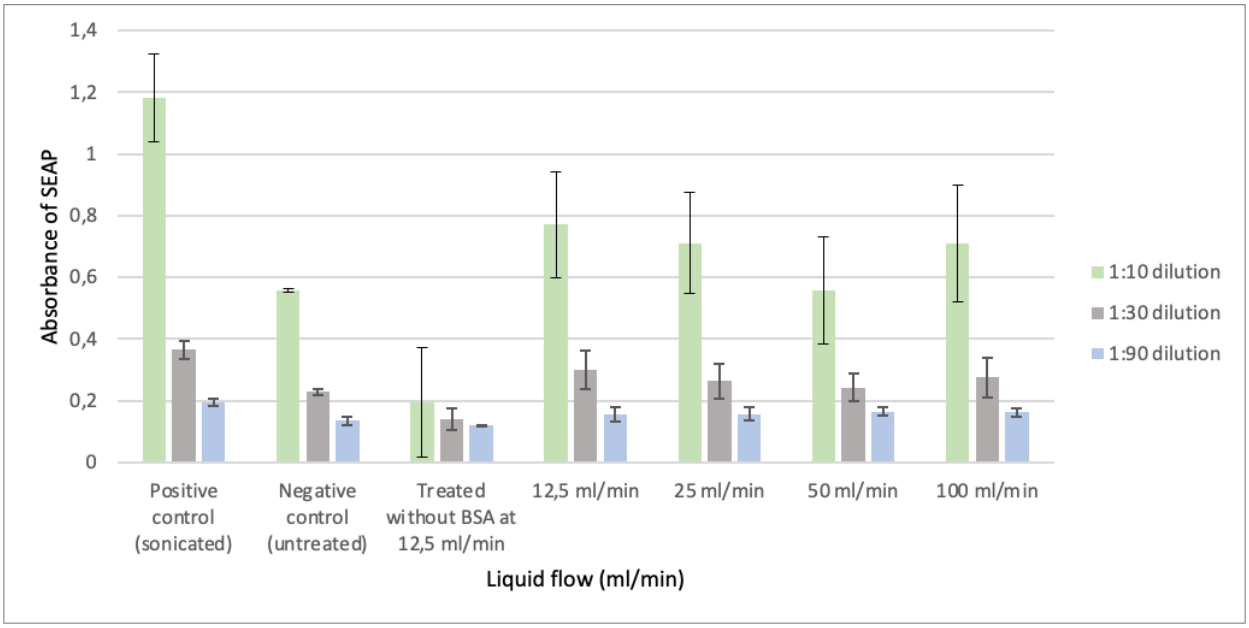

Figure 15: Measurement of endotoxins following treatment with ozone and different liquid flows. Absorbance of SEAP at 625 nm in TLR2 cells can be seen after treatment in the ozone rigg, with different liquid flows and with addition of BSA. Dilutions of the samples that were treated can be seen.

4. Discussion

This study aimed to develop a method to detect lysis and utilize the method to investigate the potential lysing effect of ozone on *E. coli*. To be able to show this, a method was first developed to be able to detect the lysis. It was shown that the methodology together with sonication was effective and the Beta-galactosidase activity was subsequently measured in the sonicated samples, revealing that the BL21 strain and the IPTG-induced BL21 strain produced the most Beta-galactosidase (Figure 8). However, due to that the measurement was >9,99 for the non-diluted samples, the actual absorbance could not be measured in those. The IPTG-induced BL21-pUC-19 in the diluted samples, could then be seen producing the most Beta-galactosidase, and for the following trials, IPTG induced pUC-19-BL21 was utilized as a reference, using a bacterial concentration of $1,5*10^7$ CFU/ml. This was done since the absorbance at this concentration did not go above 9,99.

For the first trial with ozone, the goal was to introduce ozone directly into a falcon tube containing the bacterial suspension to ensure precise control of the reaction time as a previous study had done.[12] However, a very low amount of bacteria survived the various treatment times, and Beta-galactosidase could not be detected (Figure 9). This was differed from a previous study, that had a slight different method, as they saw that they still had a cell viability after 150 minutes of treatment with a bacterial concentration at 10^7 at an ozone concentration of 0,167 mg/min/l.[12] The reason for not detecting Beta-galactosidase even though the cells had died, could either be attributed to that the treatment time was too long, or that the concentration of ozone was too high, leading to Beta-galactosidase inactivation by the ozone. As Beta-galactosidase was not detected despite that the bacteria had died, an experiment was conducted to determine whether ozone inactivated Beta-galactosidase, as hypothesized in previous studies.[22,23] The results confirmed that Beta-galactosidase was inactivated by ozone treatment at all flow rates (Figure 10). In the following experiment a lower concentration was instead utilized, and the treatment was conducted in the ozone rigg instead of a falcon tube. The use of the ozone rig resulted in a significant reduction in treatment time, and it was anticipated that at a lower ozone concentration more Beta-galactosidase could be detected since less ozone could have the possibility to react with the cells, and the protein. The results showed that the viability could still be detected at an ozone concentration of 6,9 g/m^3 and with an absorbance of Beta-galactosidase (Figure 11). However, at a concentration of 7,5 g/m^3, which is not a significant increase from 6,9 g/m^3, no viability was detected. The lack of viability at 7,5 g/m^3 could be due to the instability of the ozone generator, and insufficient time was given for the generator to stabilize. Although the ozone detector indicated a concentration of 7,5 g/m^3, it could have been higher, leading to more cell death. It was observed during the lab work that the ozone generator could sometimes increase or decrease the set concentration even with sufficient time given for it to be stabilized. Therefore, the results may have been affected by this potential source of error.

Once the ozone concentration that would be utilized had been determined, the liquid flow was altered in the next experiment. The flow was set to start at 25 ml/min, allowing more time for ozone to react with the cells, and a finishing flow to 300 ml/min, resulting in minimal ozone-cell reaction. The results indicated that the viability was reduced by half compared to the untreated cells at a liquid flow of 25 ml/min, and the absorbance of chlorophenol red in the diluted sample ($1,5*10^6$ CFU/ml) was almost double compared to the negative control (Figure 12). These results support the hypothesis and previous research that have been made, that when bacterial cells react with ozone they lyse and Beta-galactosidase will be released. [12]

Furthermore, the optimal set-up for the ozone treatment involved using an ozone concentration of 6 g/m^3, having the gas flow at 5 ml/min and a liquid flow of 25 ml/min. Although, despite the successful development of the method and finding the right treatment parameters to lyse the bacteria, while preserving the Beta-galactosidase, there are challenges in optimizing the treatment parameters for other experimental setups and for the reproducibility. The use of a high ozone concentration or a long treatment time could lead to more cell death and Beta-galactosidase inactivation, but a lower concentration could instead not be sufficient to lyse the cells. It is therefore important to investigate these parameters to gain reproducible results. Furthermore, the human factor could also have impacted these results. All the samples were pipetted by hand and when doing dilution series, factors such as mixing, pipetting and pipetting at different time points could then have affected the results.

Following the establishment of the optimal treatment set up, efforts were made to investigate the possibility if more Beta-galactosidase could be detected by quenching any residual ozone in the samples by adding BSA. This was done as ozone can be quenched by protein, and BSA mimics the albumin that can be found in human blood. In this case the idea was to use it to inhibit any additional ozone that might be found in a sample to interact with Beta-galactosidase. The viability of the samples was not found to be affected by the presence of BSA, as indicated in Figure 13, which demonstrated no difference by comparing the samples with or without BSA. For the Beta-galactosidase absorbance, the effect of BSA was inconclusive. By comparing the sample that did have BSA and the sample that did not, at a liquid flow of 12,5 ml/min, more Beta-galactosidase can be seen in the latter sample. The sample with BSA at the same flow rate also displayed a lower Beta-galactosidase level than the negative control. The only sample that showed a higher absorbance of Beta-galactosidase was the one at 50 ml/min, but the accompanying error bars suggest that this was possibly an outlier. So, while BSA did not appear to affect the viability of the samples, its impact on Beta-galactosidase needs further exploration, since it was shown that BSA helped to keep the activity of Beta-galactosidase in the previous experiment (Figure 13) when nothing else was in the sample. This implies that additional studies are needed to be done to study the interaction between BSA and Beta-galactosidase with the interference of ozone.

Based on the indications that Beta-galactosidase gets released upon treatment with ozone, endotoxins that could potentially be released upon lysis were also investigated. Since it was shown from the results that ozone inactivated Beta-galactosidase, endotoxins were measured to investigate if these were released upon ozone treatment as a part of a lysis process. The results showed that samples that contained BSA in them displayed a higher absorbance of SEAP, hence higher endotoxin content, than the sample without BSA (Figure 15). The control sample that was treated without BSA at 12,5 ml/min has the lowest absorbance, and when comparing it towards the sample with BSA at 12,5 ml/min, it showed a much higher absorbance. One possible explanation for the higher absorbance in the sample with BSA is that the BSA has quenched the additional ozone. Since ozone can degrade endotoxins as well, the endotoxin measurement can therefore be higher in the sample with BSA. Another possibility is that the BSA contained contaminants such as other endotoxins which contributed to the higher absorbance. This theory seemed more plausible as the negative control, that had BSA in it, had a higher absorbance than the treated sample without BSA.

In conclusion, these results indicate that ozone has a lysing effect on bacteria at low concentrations, which would confirm the hypothesis. However, additional studies need to be done to confirm these results, as the protein that was analyzed was found to be inactivated by ozone at higher concentrations and the method to keep the protein active by using BSA, was not successful in this study.

5. Future Perspectives

The study successfully achieved the aim of developing an effective method to detect lysis of *E. coli* using the protein Beta-galactosidase. The method was used to detect lysis of *E. coli* by ozone, utilizing a setup of 5 ml/min in the gas flow, 6 g/m3 in ozone concentration and 25 ml/min of liquid flow. However, further investigations are needed. For example, electron microscopy could be used to examine the cell morphology pre and post treatment to verify cell lysis as done in a previous study.[12] In addition, the stability of the ozone generator should also be investigated as it may have affected the results. By using an ozone generator that is more stable, more precise ozone concentrations could be delivered and more accurate and reproducible results may be obtained. The reaction time may also be further explored. This can be done in the ozone rigg by changing the length of the reaction chamber, leading to more or less exposure time. Despite the unsuccessful attempt to detect Beta-galactosidase by adding BSA to the bacterial culture, results with only Beta-galactosidase and BSA showed promising results. Additional experiments controlling for background variables needs to be done to confirm the hypothesis and the BSA used should also be investigated for contaminations. Finally, for endotoxins, a measurement with TLR4 cells could also be done that checks for LPS and lipid A.

6.References

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