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Working Towards the Heterologous Expression of Styrene Monooxygenases for Styrene Epoxidation and Reaction Cascades

Degree Project C in Chemistry

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October 2018

Abstract

Previously, styrene oxide has been used as starting material in the reaction cascade for obtaining 2-hydroxyacetophenone. If the path could be extended to instead start with styrene as substrate, it would be an advantage financially. The aim of this degree project was to produce two monooxygenase components needed for the epoxidation of styrene. The coding sequence for styrene monooxygenase component StyA had in an earlier project been inserted in a plasmid which in this project was used for inserting the gene coding for the styrene monooxygenase component StyB. The transformation of the ligated plasmid became problematic and did not result in the expected outcome. When doing an transformation directly on the ligation mixture, the result of the experiment was successful. Consequently, the problem was likely due to the poor condition of the cells used.

Contents

Abbreviations

rpm	Revolutions per min
2TY	2X Tryptone Yeast extract, (microbial growth medium)
LB	Lysogeny Broth, (microbial growth medium)
TRIS	Tris(hydroxymethyl)aminomethane
BSA	Bovine Serum Albumin
DNA	Deoxyribonucleic acid
Bp	Base pairs
StyA	Styrene monooxygenase compound A
StyB	Styrene monooxygenase compound B
FAD	Flavin Adenine Dinucleotide
NAD^+	Nicotinamide Adenine Dinucleotide
g	Relative Centrifugal Force

1. Introduction

1.1 Green Chemistry and Biocatalysis

During the last decades, Green Chemistry has become more relevant because of the greater concern regarding the waste that chemical industries release into the environment. An alternative considering cleaner chemistry was needed and a wider use of enzymes can contribute.

Researchers discovered that microorganisms, other than yeast that had been used for some time, could be applied in useful chemical transformations. From previously using only chemocatalyst, one now started to focus more on biocatalysis, were you apply enzymes and whole microorganisms to synthetic chemistry. This works in the way that enzymes are isolated from microorganisms, such as *E. coli*, yeast etc., for use in catalytic reactions. Through protein engineering the synthesis of new synthetic intermediates was allowed, for example, the conversion of only a particular enantiomer of the molecule is possible. In the pharmaceutical industry this had an enormous impact due to that biocatalysts could be applied to obtain molecules, such as α-hydroxy ketones, that could be used in the making of pharmaceuticals. Using biocatalysts would reduce the amount of chemical waste released into the environment and thus meet one of the requirements of Green Chemistry. [1]
Through protein engineering, enzymes can be modified to obtain specific properties. This technique has contributed to the exponential increase in the number of biocatalysts in recent years. Instead of making the substrate fit the enzyme, it is now possible to construct an enzyme to fit the substrate. This has been both financially and environmentally favorable, due to the fact that enzymes are made from cheap and renewable resources and are relatively easy to come by. [2,3,4]

1.2 Bacteria

Bacteria are single celled microorganisms. The bacteria contain, in addition to chromosome and proteins, one are more double stranded circular DNAs, known as plasmids. The plasmid may provide properties, such as antibiotic resistance, that will come to benefit the bacteria in an environment with antibiotic present. An important sequence of the plasmid is the origin of replication which ensures that replication will occur.
Constructed plasmids, carrying foreign genes, can be inserted into bacteria for expression of the corresponding protein.[5]

The most frequently mentioned and used bacterium is *Escherichia coli*. Its well known genetics has made *E. coli* a good host for cloning and the expression of recombinant proteins. The strains frequently used, among laboratory technicians, are defined as the B-strains and K-strains. In the respective wild type-strains, a DNA restriction enzyme is present to cleave foreign DNA. The K-strain XL-1 Blue carries the *hsdR17* mutation. This mutation will

prevent the cleavage of cloned DNA by the restriction system, and is therefore an excellent strain used in cloning of foreign genes.[6,7,8]

1.3 Enzymes

The decomposition of some molecules may take several years without the presence of enzymes. These catalysts are proteins that catalyze chemical reactions by increasing the speed of the process without being consumed themselves. Many different types of enzymes exist performing different tasks. There are three types of different restriction enzymes, I, II and III. Types I and III are ATP dependent and cleave the DNA at random locations from the recognition sequence. Restriction enzyme II catalyze the cleavages, without using ATP, of the phosphodiester bond in the DNA, to generate a smaller fragment. Using another type of enzyme, known as DNA ligase, the fragment can be inserted into a vector (plasmid DNA) with ATP as a energy source. This process is typically used when cloning a specific gene.
The appearance of the ends of the cleaved DNA differs depending on the restriction enzyme. Sticky ends are more easily re-attachable to each other due to their overhang (base pairs that are not linked to another base pair). Blunt ends are not as easily re-attached, for the reason of not having any unpaired base pairs (*Figure 1*). [9]

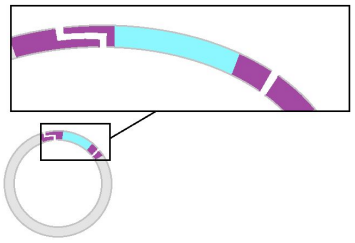

Figure 1. Digestion of a plasmid with a restriction enzyme (for example by *Sac*I) that causes sticky ends to the left and with a restriction enzyme (for example by *Sma*I) that causes blunt ends to the right.

1.4 Monooxygenase and Styrene Monooxygenase

Monooxygenases are enzymes that reduce molecular oxygen and catalyze the attachment of one oxygen to the substrate and the other oxygen to form water. Various monooxygenase families have been discovered in organisms, for instance in bacteria and fungi, and have, through protein engineering, been improved regarding their activities and stabilities etc. These enzymes are used as biocatalysts due to their properties useful for drug development and the synthesis of fine chemicals.
Many enzymatic reactions require a cofactor to perform a catalytic reaction. Monooxygenases that are using flavin as a cofactor are either a single or two component system. Two component systems consists of two proteins that work together to be able to catalyze the reaction, whereas one component systems only use one protein. Over the years several two component systems have been discovered dependent on reduced FAD as a

cofactor. These systems have been found catalyzing epoxidation and hydroxylation reactions. [10, 11]

Styrene monooxygenases (SMO) can be found among others in the gram negative bacterium *Pseudomonas putida*, were it catalyzes the epoxidation of styrene to (*S*)-styrene oxide (*Figure 4*). SMO consist of two independent homodimers (StyA and StyB), both important for the oxidation of styrene. StyA (*Figure 2*) is a 46 kDa protein subunit that uses molecular oxygen and the cofactor $FADH_2$ to attach an oxygen to styrene. StyB (*Figure 3*) is a 20 kDa protein subunit that contributes in the reduction of FAD to $FADH_2$, with assistance of NADH, to complete the cycle (*Figure 4*). [12, 13]

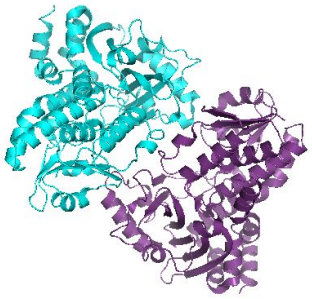

Figure 2. Structure of dimer StyA (pdb:3IHM)[14] constructed in pyMOL.

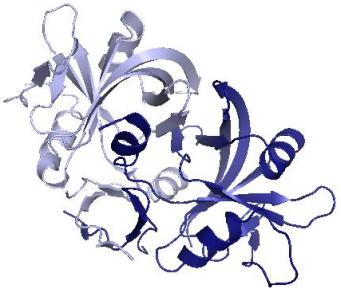

Figure 3. Structure of the StyB (pdb:4F07)[15] constructed in pyMOL.

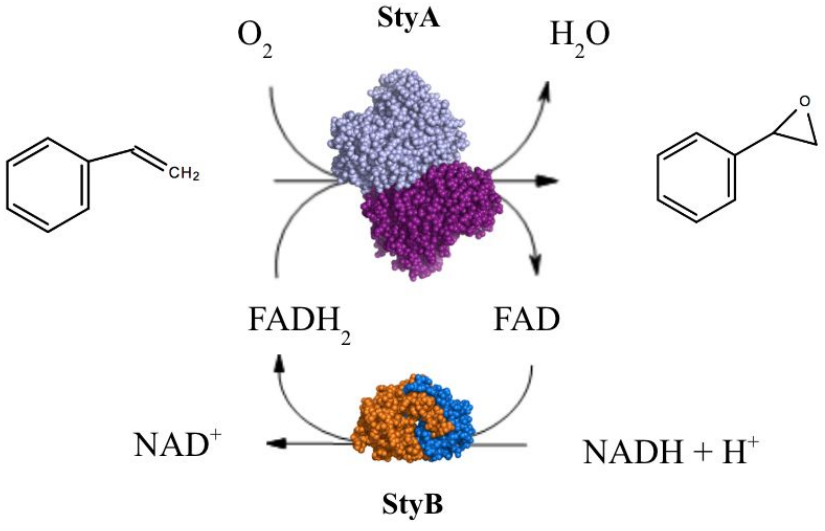

Figure 4. The catalytic cycle describe of StyA, converting styrene to styrene oxide, and of StyB, converting FAD to $FADH_2$ with NADH as electron donor.

1.5 Aim

The aim of the project was to purify the gene coding for the protein StyB and insert it into a vector (pGT7), already containing the gene coding for the protein StyA. The ligated vector would then be cloned, expressed and purified for use in the epoxidation of styrene. The substrate could then be hydrolysed to α vicinal diol, and used in the modification of an α-hydroxy ketone (*Figure 5*). If this was possible, it would be financially advantageous to use the styrene monooxygenase components a and b, since epoxides are widely used starting material for the synthesis of organic molecules.

A similar project has been performed previously, without the application of StyB. This because *E. coli* naturally has a system that reduces FAD and StyB was therefore considered not to be needed.[16] That assumption proved false when radicals were formed and that is why StyB was to be applied in the process to stabilize StyA.[16]

The goal is to get the purified StyA and StyB to work together as an independent oxidation factory, where the production of styrene oxide takes place (*Figure 4*).

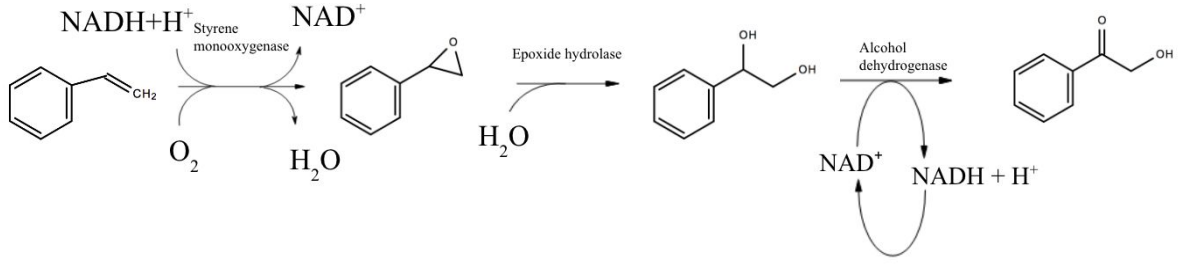

Figure 5. Styrene used as starting material for the creation of 2-hydroxyacetophenone.

2. Experimental

2.1 Construction

Using *GeneArt® (Thermo Fisher Scientific)*, a plasmid containing the gene coding for the protein StyB was constructed. The gene was devised to contain *E. coli* optimized codons and restriction sites to facilitate its insertion into the vector. In addition to the gene a sequence for a T7 promoter and a polyhistidine tag (6 His tag) were included. The polyhistidine tag was added to make it feasible to purify the protein by Immobilized Metal Ion Affinity Chromatography.

2.2 Digestion & Ligation

The first digestion of the pGT7 vector (containing the StyA gene) was done using three combinations of restriction enzymes (*Sac*I-*Spe*I, *Sma*I-*Spe*I, *Xho*I-*Spe*I) to cut out different fragments of the vector. This was done to confirm where the restriction sites of the vector are located to be able to continue with the second digestion.

The second digestion on the pGT7 vector (*Figure 6*) was performed using 100 ng of the vector, 1 µL of *Sac*I and *Sma*I and 2 µL of Buffer B (10 mM Tris-HCl, 10 mM $MgCl_2$, 0.1 mg/ml BSA) and 15.7 µL autoclaved deionized water. The digestions were incubated overnight at 37 °C and heat inactivated at 65 °C before loaded, together with 1 kbp GeneRuler ladder, onto 0.8 % (w/v) agarose gels containing 1x GelRed™ (to stain the gel). Purifying the DNA from the gel was done using the *GeneJET gel extraction kit.* The concentrations of the vector was measured using the *NanoDrop® ND-1000 spectrophotometer*.

The digestion of the StyB gene (*Figure 6*) was performed with 1 µL of the restriction enzymes *Sma*I and *Sac*I, 2.5 µL Buffer B, 1000 ng plasmid and 14.4 µL autoclaved deionized water. The digested gene was then incubated overnight at 37 °C, heat inactivated at 65 °C and purified in the same way as, the pGT7 vector.

Another digestion was performed as described above. Tango Buffer (33 mM Tris-acetate, 10 mM magnesium acetate, 66 mM potassium acetate, 0.1 mg/ml BSA) was used instead of Buffer B, to see if it would make a difference in the amount of the digested DNA. The digestions were loaded onto 0.8 % (w/v) agarose gels together with a 1 kbp GeneRuler ladder, containing 1x GelRed™ (to stain the gel).

The ligations of the StyB gene and the pGT7 vector (*Figure 6*) were carried out with 2.0-10.0 ng of the vector (digested with *Sac*I and of *Sma*I) and 10.0-31.0 ng of the StyB gene. With assistance of T4 DNA ligase and 0.4 mM ATP the gene and vector were ligated in 10 x T4 DNA ligase buffer. A negative control was performed similarly, but instead of the digested

styB it contained autoclaved MQ-water. The ligation mixture was then centrifuged, 1 min at 5,000 g in a table top centrifuge, before incubated in room temperature overnight and heat inactivated at 75 °C, for 10 minutes.

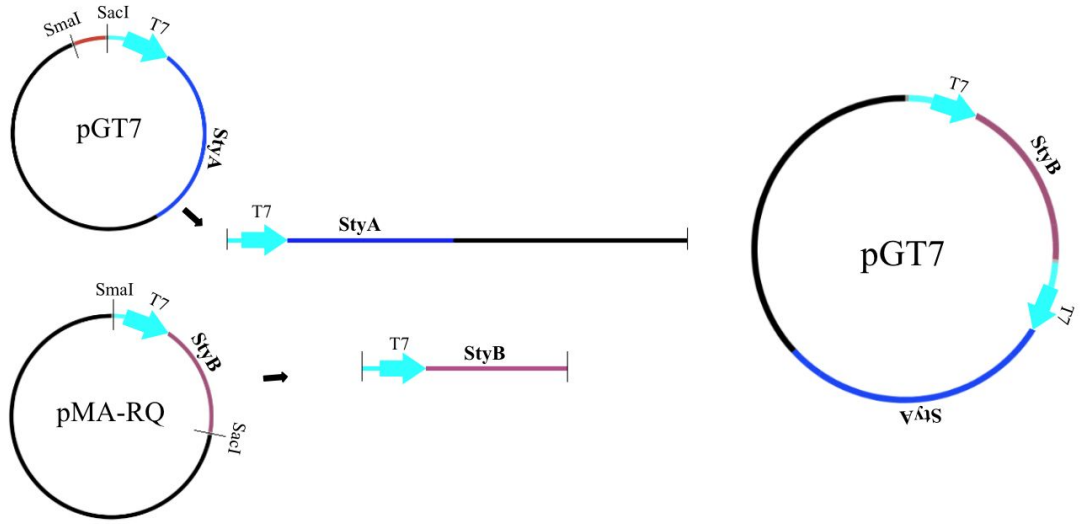

Figure 6. The digestion and ligation of the vector pGT7 with the styB gene.

The ligation mixture were then purified from unwanted salts using ethanol precipitation with 99.5 % ethanol, 3 M sodium acetate (pH 5.2) and autoclaved deionized water. Before centrifuged 30 min at 5,000 g the mixture was incubated for 1 h at -80 °C. The pellet was washed with 99.5 % ethanol and resuspended in 10 µL autoclaved deionized water.

Two additional ligations were performed as described above. One ligation mixture with ligase and one ligation mixture without. The ligations were analyzed using agarose electrophoresis, together with 1 kbp GeneRuler ladder. This step was performed to indicate if the gene and the digested vector had been ligated.

2.3 Transformation

To obtain more of the ordered plasmid (containing the gene coding for StyB) transformation was done through electroporation (1.25 kV). A negative control and one positive control were made with 60 µL of electrocomponent XL1-Blue cells, and either 1 µL MQ-water (negative control) or 1 µL plasmid à 1000 ng (positive control). After the electroporation, 1 ml 2TY medium were added and then the solutions were transferred into culture tubes to then be incubated one hour at 37 °C, 200 rpm. Before spreading 100 µL of the cells on LB agar plates (Table 1 in appendix) the solutions were diluted 10-, 100- and 1000-fold. The plates were incubated overnight at 37 °C to obtain colonies. The colonies were inoculated into culture tubes containing 100 µL ampicillin and 2 ml 2TY (table 1 in appendix). The culture tubes were incubated overnight at 37 °C, 200 rpm. With assistance of the *GeneJet plasmid*

miniprep Kit the plasmids were purified and, using the *NanoDrop® ND-1000* spectrophotometer, DNA concentrations were estimated.

From the precipitated and resuspended ligation mixture, described above, one positive and one negative control ligations was performed. 0.2 µL from various ligation mixtures was transformed into 50 µL of the XL1-blue strain of *E. coli*, to be electroporated at 1.25 kV. To the cells 1 ml 2TY medium was added and the mixture was incubated for 1.5 h at 200 rpm, 37 °C. The cells were then grown on LB agar plates, from both the negative and positive control, and incubated overnight at 37 °C. The procedure was repeated several times with different volumes of ligation mixture.

A transformation, was performed as earlier described, with 1 µL of the pGT7 vector and 50 µL XL1-blue cells to be electroporated at 1.25 kV. The LB agar plates, containing the transformation solution, were incubated at 37 °C overnight.

An transformation was performed, directly from the ligation mixture, using the BL21-AI strain of *E.coli*. The transformation was carried out with 0.5 µL of the ligation mixture and 50 µL of cells, electroporated at 1.25 kV and incubated 1h at 35°C in culture tubes, containing 1 ml 2TY media. The cells were grown on LB agar plates overnight at 37°C.

3. Results

3.1 Digestion

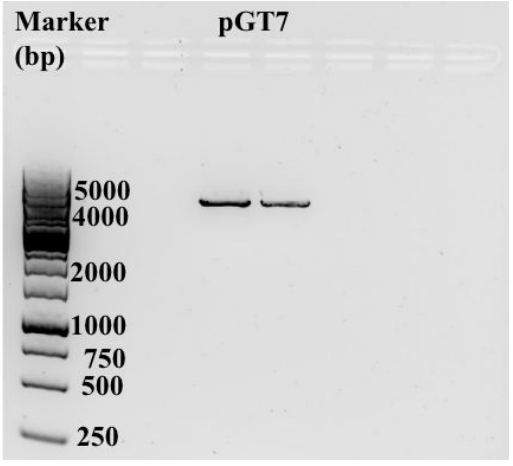

Figure 7. With restriction enzymes *Sma*I and *Sac*I the digested pGT7 vector.

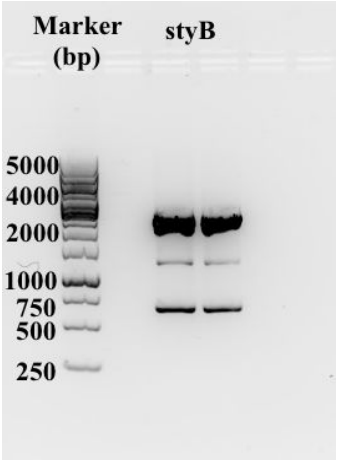

Figure 8. The styB gene digested out of the pMA-RQ plasmid using the restriction enzymes *Sma*I and *Sac*I.

The excision of the styb gene (from the purchased plasmid) and of the pGT7 vector were successful, using restriction enzymes *Sac*I and *Sma*I. This was confirmed when analysing the purified digestions in agarose electrophoresis, though a third band appeared around 1,500 bp

from the digestion of the styB gene (Figure 8). The digested band of the pGT7 vector appeared around 5,000 bp (*Figure 7)* and for the styB gene around 750 bp (*Figure 8*).

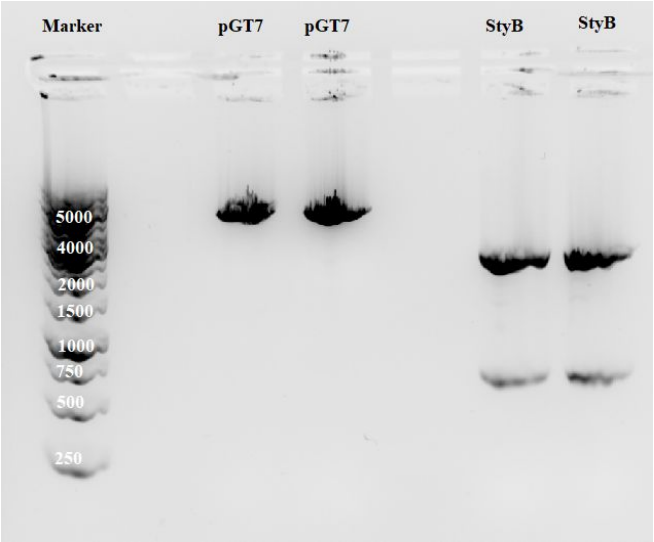

Figure 9. Digestion of the pGT7 vector and styB performed in Tango buffer, using the restriction enzymes *Sma*I and *Sac*I.

The second digestion attempt, on the pGT7 plasmid and of the styB gene, using Tango Buffer turned out to be successful, though some of the DNA appears to remain in the wells (*Figure 9*).

3.2 Ligation

The ligation of the pGT7 vector and the StyB gene were performed, unsuccessfully, a number of times. This indicated that the ligation mixture was not, when transformed onto LB-plates, giving any colonies. The concentrations of every ligation mixture were estimated and seemed relatively high. During the attempts of ligation, freshly opened ATP and DNA ligase was applied to see if the result would change.

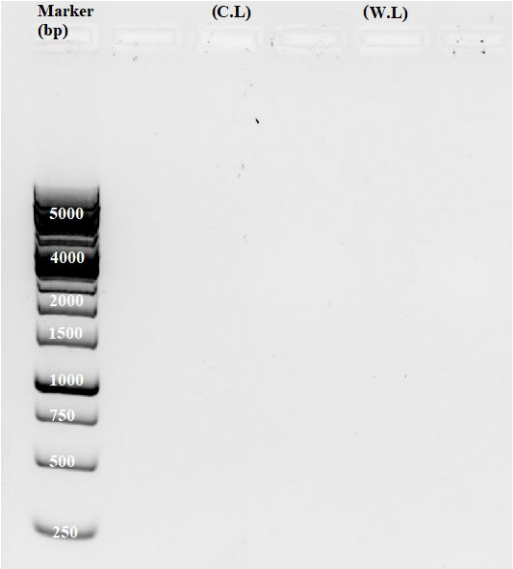

Figure 10. The ligation mixtures, one containing ligase and one made without ligase. (C.L= Containing ligase, W.L= Without ligase).

The ligation mixtures with and without ligase gave no result after loaded to the agarose electrophoresis (*Figure 10*) suggesting that the quantity of DNA was too small to be displayed on the gel.

3.3 Transformation

Several different volumes of ligation mixture were used, this because the electroporator generated a spark which indicates that the salt concentration was too high in the solutions. After washing the solution using ethanol precipitation the concentration was lowered, tho a spark was still generated from the electroporator. Therefor a smaller volume of the ligation mixture was used when transformed. The transformations of the ligation mixtures, on both those who emitted a spark and on those who did not, were unsuccessful.

Cloning on the styB gene from the purchased plasmid was done several times before a result of colonies was displayed. To find out if the styB gene was present in the cloned plasmid, an 0.8 % agarose electrophoresis with digestions from both the cloned plasmid and the purchased plasmid were performed to compare locations (*Figure 11*). A smaller band could be seen around 750 bp, suggesting that the digestion of the cloned plasmid, containing the gene styB, had been successful. The purchased plasmid seems to have an extra band around approximately 7,000 bp (*Figure 11*).

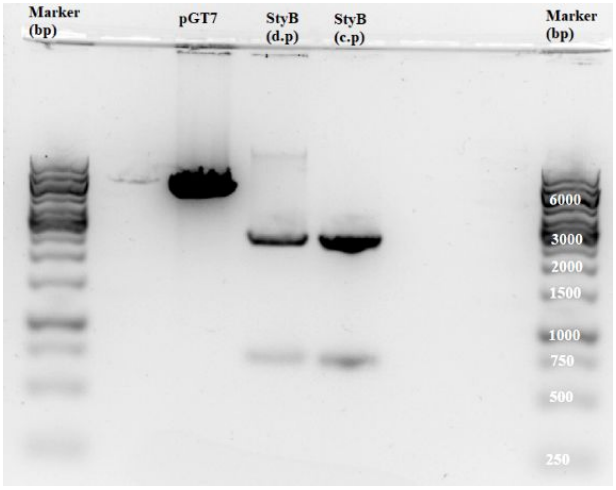

Figure 11. Analysed digested pGT7-vector, containing StyA, using the restriction enzymes *Sac*I and *Sma*I to the left of the gel and digested styB genes (d.p = purchased plasmid, c.p = cloned plasmid) using the enzymes *Sma*I and *Sac*I, the two to the right.

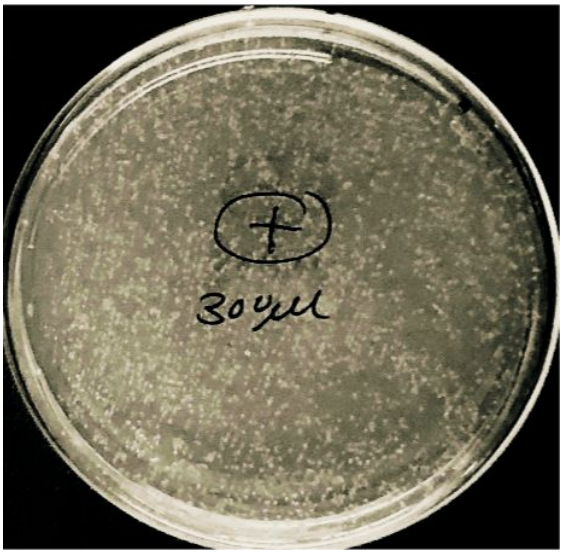

Figure 12. Colonies yielded after cloning the ligated plasmid.

The cloned ligation mixture, using BL21-AI cells, yielded colonies after incubation overnight at 37°C (*Figure 12*).

4. Discussion

It is not easy to comprehend why the cloning was not successful using the XL1-blue cells. Different aspects were considered. The ratio of the amount digested gene and vector was changed a number of times when ligated, to see if the result would change. When the outcome stayed the same another digestion was made using the Tango Buffer, but the result, displayed on the gel, were not better than those obtained when using Buffer B. Both had been digested properly, but the bands of the digestions using Tango Buffer were not as clear

indicating that to much DNA had been added to the wells and is why the outcome of the bands were not as clear as when using Buffer B. The DNA stuck in the wells, from the digestion performed in Tango Buffer (Figure 9), suggested that the comb were not clean when casting the wells. The digestion of the styB gene performed in Buffer B received a third band indicating that the solution might have been contaminated. Also, the possibility that the environment for the restriction enzymes was not optimal exists, and therefore the enzymes did not perform the digestions properly. If the digestions were performed poorly, the ligation could not be done particularly well and could be a contributing factor to why no colonies were obtained.

When measuring the concentration on the ligation mixtures, they all seemed relatively high. Using ethanol precipitation salts were removed. The concentration was lowered but the electroporator still generated a spark, suggesting that the concentration still was too high. Probably, the concentration of the DNA was to high to be able to be electroporated and should most likely have been further diluted.
Due to the high concentration of DNA in the ligation mixture, volumes used without complications from the electroporator became extremely small. By that, a possibility exist that the mixture did not enter the electrocompetent cells (XL1-blue), therefore cloning of the plasmid could not be performed and no colonies were obtained.

The ligation, performed both with and without ligase, was made to make sure that the one without would on the gel display the gene and the vector separately. When the gel was analysed, nothing could be seen except the ladder used. Not seeing any bands from neither of them indicated that the quantity of the DNA was to small to appear on the gel. This because in the ligation mixture without ligase, both the gene and the vector would have been seen. The ligations were remade with higher concentrations but did not contribute to any visible difference. Presumably, the concentration of the DNA was still too small to be seen.

In order to ensure that no unwanted contaminations had interfered, new solutions were prepared and each part was repeated once more. The result showed no difference from previously used solutions and the issue could still not be solved. Even though the ligase was newly opened there is a chance that the ligase had become bad since the expire date had passed.

One assumption considered was that the digested vector had self ligated or ligated with another vector. This was proven wrong when no colonies were obtained upon transformation into the XL1-Blue cells. Due to the fact that the vector would have been transformed in the case without the styB gene, because of the ampicillin resistant gene. Another thought was that the blunt ends, caused by *Sma*I, did not ligate properly, but could not be proven.

Most of the ligations were incubated in room temperature overnight except the last attempt (incubated around 16 °C) which also proved unsuccessful. The possibility that the

temperature would be reduced further exist, and could be tried out in a ligation attempt in the future.
Something that can also be considered is to use two separate plasmids (vectors) to ligate the fragments encoding the respective enzymes. This to see if the problem lies in the difficulty of using one vector.
Vector pGT7 (containing the gene coding for StyA) had been constructed by a previous student, why it was not necessary to order the sequence coding for StyA. The vector used may have become poorly and could be a contributing factor to why the transformation using the XL1-Blue cells did not give a positive result.

The transformation of the pGT7 vector was performed to see if the cells used were in a good condition. When colonies were displayed, it turned out that the cells presumably were not a contributing factor to the cloning. Tho, when doing an transformation on the ligation mixture, into the BL21-AI strain of *E. coli*, and colonies occurred, this was proven wrong and indicated that the ligation had been successful.
The most likely reason why the ligation did not work using the XL1-blue strain of *E. coli* was due to that the cells presumably had become bad and therefore did not provide expected result.

5. Conclusion

To conclude the project, the most likely reason why the transformation, using XL1-blue cells, did not yield colonies was probably due to that the cells were too old. The transformations that gave colonies, occurred most likely due to pure luck. If it had been discovered earlier, the change to newer cells might have given the possibility to obtain rewarding results. Furthermore, if performed in later experiments, the cells condition should be thoroughly observed before used.

6. Acknowledgements

I would like to thank my supervisor prof. Mikael Widersten for helping and giving me the opportunity to work on this project. I would also like to express my gratitude to Widerstens group for welcoming me and for all the help they have given me during this time.
Lastly, I would like to thank my friend and coworker Irena Serveta for the support she gave in the lab, and for making the frustrated days easier.

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8. Appendix

Table 1. Description for preparations of growth media.

Growth Media	Volume (mL)	Tryptone (g)	Yeast extract (g)	NaCl (g)	Agar (g)
2TY Media	300	4.80	3.00	1.50	-
LB Media	250	2.50	1.25	2.50	3.75

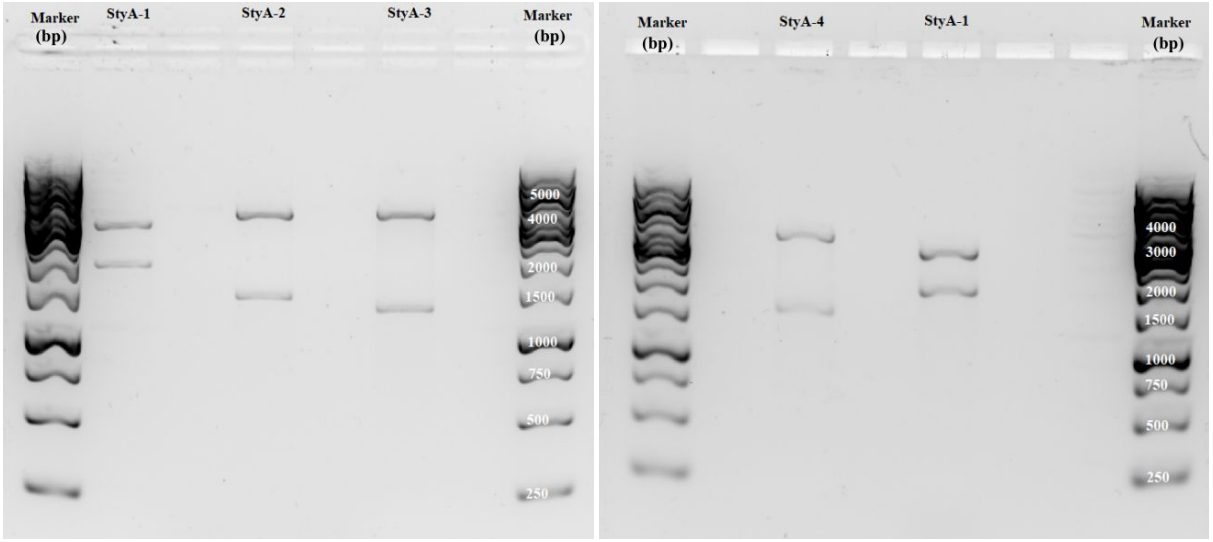

Figure 13. Digestion of the vector pGT7 with restriction enzymes to see if the restriction sites are on the known locations on the vector. StyA-1 using *Spe*I and *Sca*I, StyA-2 using *Spe*I and *Sma*I, StyA-3 using *Xho*I and *Spe*I, StyA-4 using *Sac*I and *Spe*I.