Cloning, Expression and Production of *Vicia faba* Leghemoglobin B

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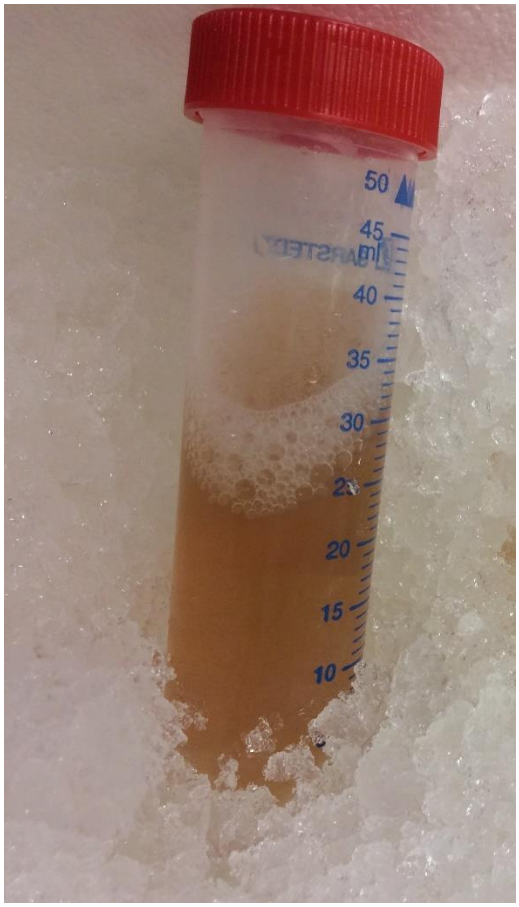

Abstract

In this thesis a preliminary evaluation of the potential of a leghemoglobin (Lb) for the development of a blood substitute in humans, resembling hemoglobin (Hb) in red blood cells, was conducted. Leghemoglobin B (*VfLbB*), a gene from *Vicia faba*, was cloned into a pET-DEST42 vector, using the Gateway™ recombination technology. The recombinant vector containing *VfLbB* was sequenced and confirmed. Then it was transformed into an *Escherichia coli* BL21-DE3 strain. Seven shake flasks experiments, with volumes ranging from 250 to 660 ml, were conducted. One experiment was dedicated to constructing a bacterial growth curve and the rest to express VfLbB. In four out of these six protein expression experiments, various parameters related to the induction of VfLbB expression were optimized. The two last shake flasks experiments were conducted with the optimized conditions. After the shake flask experiments, the production was scaled up to a five liter fermenter, where three fermentations were carried out. The first fermentation was dedicated to construct a bacterial growth curve and the other two to express VfLbB. Cells from the optimized shake flask cultivations and from the fermentations were sonicated in order to extract VfLbB. Attempts were done to purify VfLbB by carrying out ion exchange chromatography. Two different media were used: CaptoS (cation exchange) and QFF (anion exchange). Unfortunately, no positive results were obtained indicating that the purification needs to be further optimized.

Table of Contents

Purpose

The purpose of this thesis is to clone the gene of Leghemoglobin B from *Vicia faba* (VfLbB) in *Escherichia coli* BL21-DE3 in order to over-express it and purify it. The project is a preliminary stage to evaluate the potential of a leghemoglobin (Lb) for the development of a blood substitute in humans, resembling hemoglobin (Hb) in red blood cells.

Background

Hemoglobin

Hemoglobin (Hb) is a protein found in red blood cells, which are a major fraction of the blood. Its main function is to transport oxygen in the body, but it also has a function to carry hydrogen ions and carbon dioxide. In highly evolved vertebrates, Hb is a tetramer, i.e. consists of four sub-units: two α- and two β-chains. The α- and β-chains differ from each other. They, however, share a homology which is called the globin fold. Globins consist of eight α-helices with loop structures linked in between. Six of these helices are in a bundle and two are paired in an antiparallel manner. These structures altogether create an active site (Berg, et al., 2006) (Branden & Tooze, 1999).

Each sub-unit contains heme, a prosthetic group. This is located in the active site of the globin fold. The structure of heme has a centrally located iron atom, where the oxygen binds. This atom is surrounded by an organic component, which consists of four pyrrole rings. The components in heme build up a plane, in which the unoxidized iron, Fe^{2+}, does not fit within. When oxygen binds to the iron atom, it oxidizes the iron from a larger Fe^{2+} to a slightly smaller Fe^{3+} ion. This decrease in size of the iron atom enables it to fit within the heme plane and consequently the heme structure changes. Thus, hemoglobin has two forms, deoxyhemoglobin, which has no oxygen bound, and oxyhemoglobin, which has the oxygen bound (Berg, et al., 2006).

Moreover, a proximal histidine is linked to the heme-group. Oxygen is bound to Hb in a cooperative manner between the four sub-units. This means that once the first oxygen molecule has been bound to the first sub-unit, the binding of the next molecule is facilitated to the second sub-unit, followed by the third and the fourth (Berg, et al., 2006). Hb has a 250 times greater affinity for carbon monoxide (CO) than for oxygen. Consequently, Hb binds carbon monoxide

instead of oxygen when it is present and this gives rise to hypoxia, followed by death (Kim & Greenburg, 2013).

Another oxygen carrying protein in the human body is myoglobin (Mb). The main function of Mb is to store oxygen. It consists of one sub-unit which is structurally similar to the sub-units of Hb, i.e. also contain a globin fold with heme (Berg, et al., 2006). In humans, two Hbs exists: adult Hb (AHb) and fetal Hb (FHb). FHb is formed in fetuses and its oxygen affinity is higher than AHb. Due to the higher affinity, oxygen transport to the fetus is facilitated during pregnancies (Alberts, et al., 2007). A single mutation in one of the two α-chains gives rise to this affinity difference (Kim & Greenburg, 2013).

Using Hb as a blood substitute has been a research phenomenon since the end of the 19th century. This field is now known as hemoglobin based oxygen carriers (HBOC). Introducing free Hb into the bloodstream has, however, throughout the years, showed toxic effects. Free Hb is, due to its small size, filtered out by the kidneys and it is also toxic to the nephrons. Furthermore, being encapsulated by red blood cells seems to be a crucial factor for Hb. The reducing enzymes found inside the red blood cells seems to be more prominent than those found in blood plasma, e.g. toxic reactive oxygen species and also the non-oxygen binding methylated Hb tend to form in plasma. Crossing the blood vessel barriers is another drawback of having Hb in a free form. To prevent these toxic effects, research dedicated to modify the Hb molecule, e.g. PEGylation, have been conducted (Kim & Greenburg, 2013).

Plant hemoglobins

Hb also exists in plant species and there are three variants: non-symbiotic (nsHb), truncated (trHb) and symbiotic (sHb). Among these three types of plant Hbs, functional and structural studies of nsHbs and trHbs have not been widely conducted, in contrast to sHbs.

SHbs are present in root noodles of plants. These noodles are in symbiosis with the nitrogen-fixing bacteria genera rhizobia (Hoy & Hargrove, 2008). The rhizobia bacteria convert the atmospheric nitrogen to ammonia via a nitrogenase enzyme and consequently the plant is able to grow in nitrogen limiting soil. The extent of converted nitrogen is dependent on the amount of expressed sHb (Willey, et al., 2010). Due to the fact that the concentration of free oxygen must be kept low for the nitrogenase in order to function, the sHb have a high oxygen affinity, i.e. low dissociation and high oxygen association rates (Hoy & Hargrove, 2008).

In nsHbs and trHbs, this symbiotic relationship does not occur. These are located in multiple parts in the plants, including in the noodles. Expression of trHbs and nsHbs are induced by various factors, e.g. hypoxia and hormone signals (Hoy & Hargrove, 2008). Leiva Eriksson et al. (2014) showed e.g. that nsHbs in sugar beets are expressed differentially. sHbs and nsHbs are similar in a manner that they both have the conventional globin fold structure. TrHbs, on the other hand, have a truncated fold structure (Hoy & Hargrove, 2008). Similarly to the typical fold structure, there are two antiparallel α-helices, but the bundle is built up from four α-helices rather than six. Thus, the structure is truncated and slightly shorter in amino acid sequence (Milani, et al., 2001). The oxygen affinities among nsHbs differ and they are divided into two groups: class I and class II. The first class has higher oxygen affinity than the second (Hoy & Hargrove, 2008).

Leghemoglobins

The plants that usually contain sHbs are the family *Leguminosae*, i.e. legumes. Thus, the most common definition of sHbs is leghemoglobins (Lb) (Hoy & Hargrove, 2008). Lbs are monomeric and includes heme, hence structurally similar to Mb (Vinogradov & Kapp, 2012). In order to supply a microaerobic environment to the nitrogen fixing bacteria, which is required by the nitrogenases, the oxygen affinity of Lbs is high (Willey, et al., 2010). Compared to nsHbs, which vary in oxygen affinities, different kinds of Lbs have similar affinities (Vinogradov & Kapp, 2012).

Recombinant leghemoglobins have been produced from several legumes, including soybean and *Lupinus luteus*, yellow lupine. To enable over-expression of the Lbs, these experiments used an isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible promoter in their expression systems, *E. coli* BL21-DE3 (Hoy & Hargrove, 2008) (Kundu, et al., 2004) (Sikorski, et al., 1995) (Prytulla, et al., 1996). Experiments of other recombinant Hbs have shown that expression levels and yields can be enhanced, e.g. Ratanasopa (2015) demonstrated that by adding CO and δ-aminolevulinic acid (Ala) when producing human FHb. Ala is involved in the synthesis of heme and can be depleted.

Soybean Lb is the most studied Lb. Hargrove et al. (1997) applied site-directed mutagenesis to His61 soybean Lb, in order to evaluate how the oxygen binding kinetics was affected. Hargrove et al. (1997) also compared different spectra of soybean Lb in different conformation states. The maxima for the oxy-state, $Fe(II)O_2$ and the carbon monoxide state, Fe(II)CO, occur at different

wavelengths: 409 and 416 nm. Deoxygenation of Hbs can be achieved by introducing sodium dithionite (NaD) to the molecules. This method is beneficial because the protein structure is preserved (Wireko & Abraham, 1992).

Vicia faba – broadbean

Vicia faba, also called broad bean, is a legume of high popularity in Europe and Middle East. In Sweden, it was a common source of food until the 1940s and is now gradually making a comeback (Oregon State University, 2010) (Wikipedia, 2016). There exists several Lb genes in *V. faba* and they are all expressed in different amounts in the legume noodles (Kuhse & Pühler, 1987) (Perlick & Pühler, 1993). The VfLbB is a protein with a size of 15.8 kDa and a pI of 7.7, which is close to the pI of human Hb (Frühling, et al., 1997). Its oxygen affinity has not been determined and there is no extinction coefficient available.

Recombination based cloning

In this project, the Gateway® Technology will be used in the gene cloning phase. This is a cloning strategy that solely relies on recombination, i.e. no restriction enzyme or ligase is needed, and is derived from the recombination system in the lambda phage (Invitrogen (a), 2010).

The DNA fragments involved in this technology are the gene of interest, i.e. *VfLbB*, a donor vector and a destination vector. All these include attachment (att) recombination sites. There are two recombinations in this technology: the BP and the LR reaction. Firstly, the gene of interest recombines with the donor vector, pDONR, creating an entry clone. Secondly, the entry clone recombines with the destination vector, pET-DEST42, creating the expression clone. The first reaction is catalyzed by the BP Clonase™ II and the second by the LR Clonase™ II. After each of the two recombination reactions, the resulting DNA is transformed into competent cells, followed by selection on LB plates. The donor vector has a kanamycin resistance gene and the pET-DEST42 vector has an ampicillin resistance gene. Furthermore, the pET-DEST42 vector has a T7-lac promoter (Invitrogen (a), 2010) (Invitrogen (b), 2010).

Material and methods

In this section a detailed methodology of the experiments will be presented. The list and details of the equipment used is presented in appendix 1. The experiments were conducted at the Division of Pure and Applied Biochemistry at Faculty of Engineering, Lund University.

Choice of gene

The Division of Pure and Applied Biochemistry at Faculty of Engineering, Lund University, aimed to produce a recombinant leghemoglobin. The underlying decision to choose the *VfLbB* gene was based on discussion with responsible persons at the division. These discussions were based on previous research conducted in the field. The main reasons to choose the *VfLbB* gene were that its protein has not been produced recombinantly before and that it has a pI which is close to the one of human Hb.

Gene cloning

Plasmid cloning

The purpose of conducting the plasmid cloning step was to obtain a high initial plasmid concentration. *VfLbB* (GenBank Accession No. Z54157 (NCBI, 2005)) was custom synthesized by Epoch Biolabs and was obtained within a plasmid, containing attB recombination sites, pCloneEZ-VfLB (Fig. 1). 2 µl of plasmid (50 ng/µl) was transformed into *E. coli* TG1 cells (200 µl) using heat-shock transformation. First, the cells were incubated together with ice for 30 min. Then, they were heated in a 42ºC heating block for 90 seconds and subsequently cooled down on ice for approximately one minute. Afterwards, 800 µl of LB media was added and the tube was horizontally shook for 60 min at 37ºC and 225 rpm, from this 200 µl was plated onto LB plates, containing 100 µg/ml ampicillin. After the plates had dried, they were inverted and kept overnight in a 37ºC incubator. A positive and a negative control were also plated. Five colonies were picked and transferred into a glass tubes each containing 5 ml LB media plus ampicillin (100 µg/ml). These tubes were cultivated 16 hours in a horizontally shaking incubator, 150 rpm, at 37ºC.

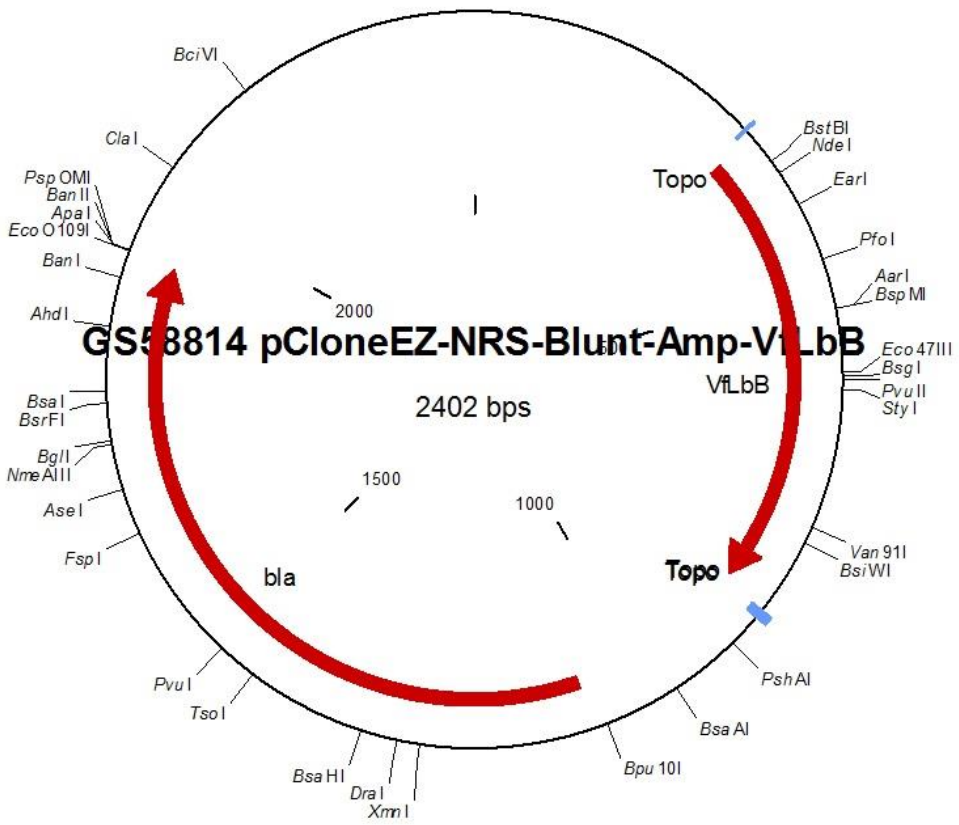

Figure 1. pCloneEZ-VfLb, the original plasmid. The blue bars in the right side of the figure indicate the location of the VfLbB gene and the PvuI restriction site is located at position 1493 (Epoch Biolabs, 2016).

The pCloneEZ-VfLB plasmid was isolated from the cells using the E.Z.N.A. ® Plasmid DNA Mini Kit I. The extraction was done according to the manual with two exceptions. First, the membranes of the used columns were activated with 100 µl 3M NaOH; second, the plasmids were eluted with 50 µl of sterile MilliQ water. The plasmid was eluted twice with the same 50 µl in order to obtain a higher plasmid concentration. The concentrations of the samples were measured spectrophotometrically and the DNA fragments were separated on a 1.5% agarose gel at 110 V. The remaining pDNA samples were stored at -18^{o}C.

Digestion and restricted plasmid recovery

Three samples of the purified plasmids were cut by the restriction enzyme *PvuI* (Table 1). This enzyme cuts once at position 1493bp, outside the positions *VfLbB* occupies (327 to 849bp) (Fig. 1). The online tool Webcutter 2.0 was used to find the enzyme (Heiman, 1997). Thus, the whole plasmid was linearized. The reactions were set up as showed in Table 1.

Table 1. Digestion reaction with PvuI.

Sample	DNA (µl)	10x buffer (µl)	Autoclaved H_2O (µl)	Enzyme (µl)	Total volume (µl)
1	27.9	3.5	2.6	1.0	35
4	24.2	3.0	1.8	1.0	30
5	16.2	2.0	0.8	1.0	20

The three reactions were incubated in a heat block at 37ºC for 30 minutes. The digested plasmids mixed with loading dye 6X were loaded on two 1.5% agarose gels with 110 V. In the first gel, to visualize the results, 5ul plasmid was used. The rest of the samples were loaded on the second gel in order to recover only the linearized plasmid containing *VfLbB* from the agarose.

This recovery was carried out according to the instructions in the manual of Nucleospin® Gel and PCR Clean-up with some exceptions. The linearized plasmids were eluted with 25 µl of sterile MilliQ water, this was repeated twice. The concentrations of the linearized plasmids were measured spectrophotometrically and then stored at -18ºC.

Recombinant plasmid construction

BP reaction

The main purpose of this reaction is to create the entry clone pDONR-VfLbB. *VfLbB* will recombine with the donor vector (pDONR). The first linearized plasmid containing *VfLbB*, was mixed on ice with the following components, supplied by Invitrogen™, see table 2:

Table 2. Components in the BP reaction, BP clonase enzyme not included.

Component	Sample 1	Sample 2	Positive control	Negative control
DNA (79.2 ng from 16.9 ng/µl)	4.7 µl	4.7 µl	-	4.7 µl
pDONR	1 µl	1 µl	1 µl	1 µl
pEXP7-tet	-	-	2 µl	-
TE buffer	2.3 µl	2.3 µl	5 µl	4.3 µl

To calculate of the amount of DNA needed in BP reaction, equation 1, was used:

$$m = (fmol)(N)\left(\frac{660\ fg}{fmol}\right)\left(\frac{1\ ng}{10^6\ fg}\right) \quad (1)$$

where m is the mass in ng. 50 fmol was used, since an equimolar relation to the donor vector would be used. The letter N stands for base pair and is the number of base pairs in the linearized fragment, derived from the digestion. The calculated required amount of DNA was 79.2 ng.

After these components were mixed, 2 µl of BP Clonase™ enzyme was added to the two samples and the positive control. The reaction mixtures were then incubated in a heating block for 16.5 hours at 25°C. The reactions were stopped by adding 1 µl Proteinase K to each of the tubes. All the resulting reaction mixtures were used to transform each 55 µl of One Shot® OmniMAX™ 2 T1 Phage-Resistant *E. coli* cells according to the instructions given in the Gateway clonase II manual (Invitrogen (a), 2010).

The transformed cells were spread onto LB plates, which had kanamycin (50 µg/ml). These plates were then incubated for 17 hours at 37°C. Five colonies, probably containing *VfLbB* inserted to pDONR, were picked and transferred into five glass tubes containing 5 ml LB media and 50 µg/ml kanamycin. These tubes were cultivated 17 hours in a horizontally shaking incubator at 37°C and 150 rpm. The plasmid of these cultivations were extracted as described above. The concentrations of the Entry Clones (pDONR-VfLbB), were spectrophotometrically measured and the DNA fragments analyzed on a 1.5% agarose gel with 110 V.

LR reaction

The main purpose of this reaction is to create the final clone, pET-DEST42-VfLbB. For this pDONR-VfLbB will recombine with the destination vector (pET-DEST42). The components of the LR reaction, supplied by Invitrogen™, were mixed according to Table 3 and kept on ice.

After these components were mixed, 2 µl of LR Clonase™ enzyme was added to the two samples and the positive control. The reaction mixtures were then incubated in a heating block for 16.5 hours at 25°C. The reactions were stopped by adding 1 µl Proteinase K to each of the tubes and then transformed into 55 µl One Shot® OmniMAX™ 2 T1 Phage-Resistant Cells according to the instructions given in the Gateway clonase II manual (Invitrogen (a), 2010).

The transformed cells including the positive and the negative controls were spread onto LB plates containing ampicillin (100 µg/ml). These plates were then incubated for 17 hours at 37°C.

Table 3. Components in the LR reaction. The LR enzyme is not included.

Component	Sample 1	Sample 2	Positive control	Negative control
Entry clone (150 ng from 307 ng/µl)	0.5 µl	0.5 µl	-	0.5 µl
Destination vector	1 µl	1 µl	1 µl	1 µl
pENTR	-	-	2 µl	-
TE buffer	6.5 µl	6.5 µl	5 µl	8.5 µl

Five colonies were picked and cultivated in Lb media. The plasmids were purified as before and a sample was sent for sequencing to GATC Biotech, Germany. Once the gene sequence was confirmed, 1.22 µl pET-DEST42-VfLbB (82 ng/µl) was transformed into 200 µl *E. coli* BL21-DE3 as described before. Three similar but independent transformations were carried out and then plated onto LB plates containing ampicillin (100 mg/ml). The colonies obtained were later used for the shake flask experiments and the fermentations.

Shake flask cultivations

Shake flask cultivations were conducted with different purposes. One cultivation was dedicated to construct a bacterial growth curve, four to optimize crucial protein expression parameters and two to express VfLbB under the optimized conditions. These cultivations were conducted in two different shake flask sizes: 2L and 1L.

The 2L flasks had 600 ml terrific broth media (TB) completed with 60 ml potassium buffer (0.72 M K_2HPO_4 and 0.17 M KH_2PO_4) and the 1L flasks had 225 ml TB completed with 25 ml potassium buffer. Furthermore, 660 and 250 µl carbenicillin (100 mg/ml) were added to the 2L and 1L liter flasks, respectively.

For the pre-culture, one colony was inoculated into 10 ml LB plus ampicillin (100 µg/ml). It was cultivated overnight at 30°C and 150 rpm. The next day, 0.5 ml and 1.5 ml pre-culture was added to the media in the 1L and 2L shake flask, respectively.

The expression of VfLbB was induced with IPTG and Ala. After induction, the temperature was decreased to 22^{o}C and the incubator was covered to keep the flasks in darkness. The cells were incubated overnight after induction and harvested the next morning.

For harvesting, 250 ml centrifuge bottles were used. These were filled with around 200 ml media and centrifuged for 10 min. at 10000 rpm. The replicates of each experiment were harvested together, unless the color of the pellets where very different within a condition. This was usually decided by pre-evaluating the color of the pellet in 1.5 ml Eppendorph tubes. This pellet, was a result from 4.5 ml culture taken from each flask and centrifuged three times at 10 000 rpm for 1 min each time discarding the supernatant in between.

The pellets of the 250 ml bottles were then transferred to 50 ml Sarstedt tubes. This transference was aided by the addition of 5 ml lysis buffer. The 50 ml tubes, containing the pellet, were centrifuged for 15 minutes at 12 000 rpm, the supernatants were discarded, and the tubes inverted over paper to absorb all the liquid. The wet paste weight was determined and then the tubes were snap-frozen in liquid nitrogen and stored at -80^{o}C.

Growth curve of *E. coli* with pET-DEST42-*VfLbB*

The first experiment was to determine the growth curve of E. coli carrying the recombinant plasmid. Three independent cultivations in 2L liter shake flasks, each with a total volume of 660 ml, were carried out. In this experiment optical density measurements at OD_{600} were taken every hour for 31 hours with an exception between hour 14 and 23. The expression of VfLbB was not induced. From this experiment, the specific growth rate and generation time were calculated to be 1.37 h^{-1} and 30.3 minutes, using the formulas presented in appendix 6.

VfLbB expression: initial optimization

A first experiment was carried out under pre-determined conditions as initial point in order to see if VfLbB was expressed. The cultivation was done in 2L flaks, and the cells were induced after six hours of cultivation at OD_{600} 3 ± 0.5. IPTG and Ala were added to a final concentration of 0.5 mM and 0.3 mM, respectively and the cells were bubbled with CO (20-30 sec). The entire cultivation was done at 150 rpm and in triplicate.

Since the addition of CO helps the heme to be attached to the globin apo-protein, the time of addition of this gas was tested. Thus CO was added by the time of induction or just after harvesting the cells. For this, VfLbB was cultivated in 1L shake flasks and its expression induced

at OD_{600} 0.8 and 1.5. Additionally, two different stirring speeds were tried during the entire cultivation, 80 and 150 rpm. The flasks were induced with 0.5 mM IPTG and 0.3 mM Ala. Each condition described here was carried out in duplicate. In total sixteen flaks were cultivated (Appendix 2, Tables 12-22).

Time of Induction optimization

VfLbB was induced at three different OD_{600}. At the beginning of the cultivation, 1.0 and 2.0. In this case, CO was also added either by induction or harvesting. The concentration of IPTG and Ala was the same as in the initial optimization. The stirring rate was 150rpm. These experiments were carried out in 1L shake flasks and triplicates. Each of the six different conditions was done in triplicates (Appendix 2, Tables 23-30).

IPTG concentration optimization

Four different IPTG concentrations were investigated: 0.1, 0.3, 0.6 and 0.9 mM. The OD_{600} for induction was between 1.5 and 2.0. A final Ala concentration of 0.3 mM was used and CO was added by induction. This experiment was done in 1L shake flasks and in triplicates for each IPTG concentration.

Multiple OD_{600} samples were taken during the cultivation and to enhance the accuracy as well as compensate for the decrease in volume, the volume which the calculated IPTG concentrations was based on was 240, rather than 250, (Appendix 2, Tables 31-36).

Ala concentration optimization

By induction, which was when the OD_{600} was in the interval of 1.5 to 2.0, four different Ala concentrations were tested: 0.1, 0.3, 0.6 and 0.9 mM. IPTG was added to a final concentration of 0.3 mM and CO was added by induction.

The entire cultivation was stirred at 150 rpm. Each condition, was done in triplicates in 1L shake flasks. The volume for final ALA concentration was corrected as with IPTG optimization to 240ml. The induced amount of IPTG was 72 µl 1 M IPTG (0.3 mM), the stirring rate was 150 rpm, (Appendix 2, Tables 37-42).

Optimized conditions

Two separate cultivations were conducted with the optimized conditions from previous shake flask experiments. The induction was done at OD_{600} between 1.5 and 2.0, CO was added by

induction and the stirring rate was 150rpm. IPTG and Ala were added to a final concentration of 0.3 and 0.9 mM, respectively.

The volume was corrected to 650ml/flask as previously done with IPTG and Ala concentration optimizations. The cultivation had a total of 4L equally distributed in six 2L shake flasks, (Appendix 2, Tables 43-45).

Sonication

The frozen cells were thawed in water baths at room temperature. To normalize the protein content, the volume of lysis buffer was twice the pellet weights, e.g. six ml buffer was added to three grams of cells. The cells were completely resuspended in the buffer by vortexing. The cells were then sonicated with a two second ON pulse and three second OFF pulse with an output frequency of 20 kHz. The sonication amplitude of the samples was ranging from 40 to 65%, going from lower to higher.

After sonication, the lysates were centrifuged at 12 000 rpm, 4^{o}C, for 40 minutes, followed by transferring the supernatant, crude extract, to new tubes.

VfLbB expression analysis

CO essay

The crude extract was spectrophotometrically analyzed to determine the leghemoglobin content. Lysis buffer was used as blank and for dilution of the samples. A first absorbance of the sample was taken and then a second one after the addition of a few grains of NaD and bubbling with 1 ml of CO gas.

To obtain a quantitative measure of VfLbB in the crude extract a three-point drop correction was used. This correction uses an interpolation concept between those adjacent wavelengths, according to figure 2 and equation 2, note that it is essential to use the equation together with the figure (Owen, 1996). In this correction, two absorbance values at appropriately chosen wavelengths are taken into account, except from the analytical absorbance, i.e. VfLbB at approximately 420 nm. Although, a high absorbance is reached, there may be interfering background of contaminants to a various extent and this correction method corrects for this background.

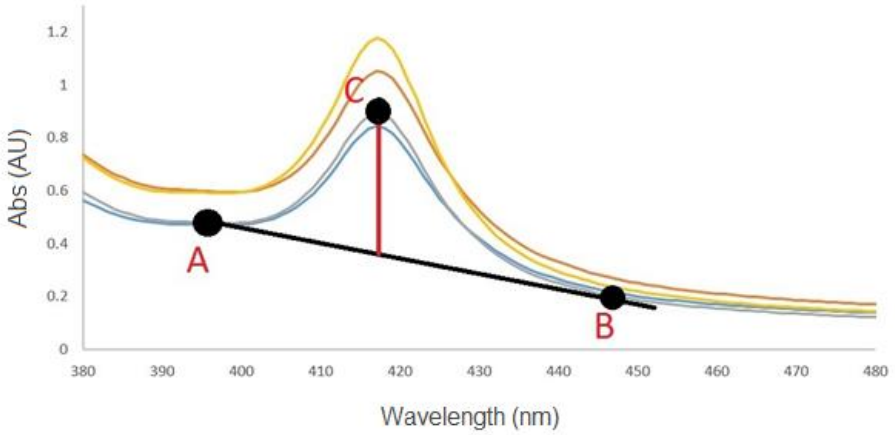

Figure 2. Three-point correction.

$$Real\ absorbance = C - \frac{A+B}{2} \quad (2)$$

In the initial optimization, the Lb content was compared using real absorbance volume per grams of cells (AbsV/W). In the following expression analyses the extinction coefficient from Lb of *L. luteus* at wavelength 417 nm and pH 8.5 was used. This coefficient is 190 mM^{-1} cm^{-1} and enabled conversion of the real absorbance values to concentration units (Fasman, 1989). From the concentration, c, milligrams of VfLbB per gram of cells (mg LbB/gr) was used as the evaluation unit.

These calculations were conducted according to formula 3-4:

$$c = \frac{A}{\varepsilon * l} * DF \quad (3)$$

$$m = \frac{(c*M)}{1000} * V \quad (4)$$

Where A stands for real absorbance, ε, the extinction coefficient, l the cuvette length, DF, the dilution factor, m, the mass in mg, M the molecular weight of VfLbB and V the volume of the VfLbB sample. The cuvette length was 1 cm.

Fermentation

Three fermentations were conducted: one was dedicated to construct a bacterial growth curve and two to express VfLbB. The fermentations were conducted according to a fermentation

protocol (Leiva Eriksson, 2016). In this protocol there are also recipes for the used solutions and media.

Media and pre-culture

A pre-culture was made by transferring one colony into a 10 ml LB tube containing 100 µg/ml ampicillin. The pre-cultures were cultivated between 8 to 14 hours at 30ºC and 150 rpm- This pre-culture was added into a 300 ml DM starter which was incubated for another 11 to 15 hours at 30ºC and 150 rpm. The DM starter was inoculated to the fermenter containing three liter DM media. The fermentations were conducted in fed-batch mode with a gradual addition of 70% glucose solution. The feeding profiles were manually written with percentage as the main unit. One percent is equal to 10 ml/hour.

Growth curve of *E. coli* with pET-DEST42-*VfLbB*

OD_{600} samples were taken every hour, except from a break between hours 14 to 23, see appendix 4. Using the formulas in appendix, the specific growth rate and generation time were calculated to 0.32 h^{-1} and 2.14 hours. The oxygen sensor did not work during this fermentation and therefore parameters such as the base, pH, and glucose concentration had to be carefully observed. Therefore, the glucose profile for the growth curve was changed multiple times, see appendix 4.

VfLbB expression in fermentor

Two fermentations were carried out where the two different OD_{600} were tested: 30 and 50. These values was obtained by graphically comparing the logarithmic bacterial growth curves from fermenter and shaking flasks (Fig 8 and 10). The bacteria were induced with 0.3 mM IPTG and 4 mM Ala. Before induction, the temperature was 30ºC, and after it was changed to 22ºC. Also the oxygen was set to 2.5% and the cells were protected from light. Compared to the fermentation where the growth curve was generated, the oxygen sensor worked and the two fermentations could thus rely more on automation, see appendix 4 for parameter details. The cells of fermentation two and three were harvested and independently lysed in a similar manner as the shaking flasks.

Purification

The purpose of this phase is to partially separate VfLbB from other molecules. One sample from the shake flasks experiments with optimized conditions and the two samples (OD_{600} 30 and OD_{600} 50) from the fermenter were purified. After lysing, the resulting crude extracts were

dialyzed. The three samples were split into half each, i.e. six samples, and dialyzed against to two different four liter buffers: NaP (Na_2HPO_4-NaH_2PO_4) 10 mM pH 6.0 and Tris-HCl 50 mM pH 8.5. Due to the fact that the molecular weight of VfLbB is 15.8 kDa, the dialysis membranes were chosen to have a molecular cut off weight of 6-8 kDa. The dialysis was conducted for 17 hours at 4^{o}C with continuous stirring.

The clarified samples were analyzed by the CO essay to confirm the LbB activity. Cation (CaptoS) and anion (QFF) exchange chromatography were then conducted. The CaptoS used a 10 mM NaP pH 6.0 solution as equilibrium and binding buffer. The elution buffer was 70 mM NaP pH 7.8. Regarding the QFF, a 50 mM Tris-HCl pH 8.5 solution was used as equilibrium and binding buffer. For the elution, a 50 mM Tris-HCl pH 7.8 buffer with 0.1 M NaCl was used.

Results and discussion

In this section the experimental results and discussion will be presented. The results are in chronological order, i.e. from the cloning phase, to shake flasks and fermentation. Additional data, e.g. sub-results are presented in appendix 1-5.

Gene cloning

pCloneEZ-*VfLbB* cloning and linearization

Bacterial colonies appeared onto the LB plates. Moreover, colonies were absent on the negative control and present on the positive one. The plasmid purification results from the - plasmid cloning are presented in table 4 and its corresponding gel in figure 3. Among the five samples, sample three had a low A260/A230 ratio, which means that it had contamination of organic components, ethanol in particular. A possible reason why this ratio was low, could be that the drying step in the plasmid purification was too short. The A260/A280 ratios were high, which means that the samples had low protein contamination. Furthermore, when observing the gel, the plasmids in sample two do not seem to be entire. Thus, because of the low A260/A230 ratio in sample three and non-entire plasmids in sample two, sample one, four and five were decided to be used in the following experiment, the *PvuI* restriction enzyme digestion.

Table 4. Sample concentration and absorbance ratios after the plasmid purification of pCloneEZ-VfLbB. Samples 1, 4, and 5 were chosen to be digested with PvuI.

Sample	[DNA] ng/µl	A260/A280	A260/A230
1	35.8	1.946	1.532
2	45.7	1.917	1.586
3	87.0	1.862	0.941
4	41.3	2.202	1.627
5	61.6	1.968	1.425

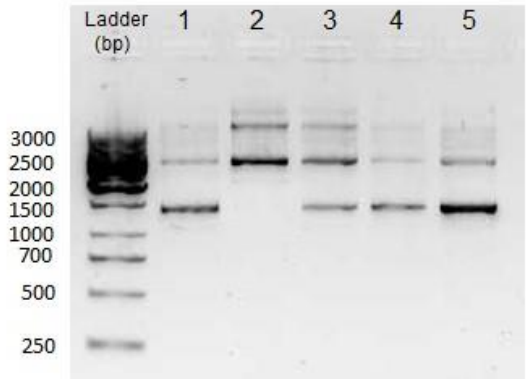

Figure 3. Purified pCloneEZ-VfLbB, after plasmid cloning.

The results from the PvuI digestion are presented in table 5 and figure 4. All the three bands are located below bp 2500, which concludes that the digestion was successfully conducted, since the size of the linear fragment is 2402 bp (Fig. 1). There is a weak band above the digested plasmids and this is most likely representing undigested plasmids. In order to avoid contamination, this band was not extracted from the gel, only the properly restricted and linearized plasmids were extracted. In contrast to the previous plasmid purification, all the purified DNA samples had rather low A260/A230 ratios, indicating high ethanol content in the samples. As before, the reason for this could be a too short drying step. A suggestion for improvement would be to increase the drying time from one minute to two minutes. Sample one, the one with the highest A260/A230 ratio, was decided to be used in the next following experiment, the BP reaction.

Table 5. DNA concentrations and ratios, after digestion with PvuI and extraction from agarose gel. Sample 1, 4, and 5 from table 4, were used for restriction

Sample	[DNA] ng/µl	A260/A280	A260/A230
1 (1 in table 4)	16.9	2.000	0.493
2 (4 in table 4)	17.9	1.895	0.316
3 (5 in table 4)	21.4	1.792	0.361

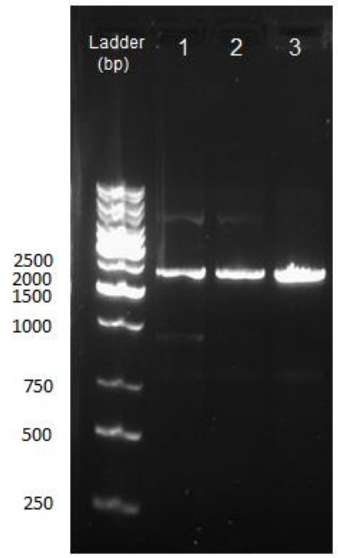

Figure 4. After digestion by the restriction enzyme.

Expression plasmid construction

BP reaction

After the BP reactions and their transformation, colonies appeared on the sample LB plates. Furthermore, the positive control had also colonies while in the negative control colonies were

absent indicating that the transformation was successful. Five colonies were picked from the LB reaction plate for further cultivation. Three of these were purified, since two of the tubes were transparent, indicating no bacterial growth.

The entry clone concentrations of the three purified samples are presented in table 6 and the results from its corresponding gel in figure 5. This purification was successfully conducted, since all three samples had high DNA concentrations and absorbance ratios. Sample two was decided to be used in the following experiment, the LR reaction, since it had the highest A260/A230 ratio.

Table 6. Entry clone concentration measurements and ratios. Sample one from Table 4 was used for BP reaction.

Sample	[DNA] ng/µl	A260/A280	A260/A230
1	460	1.896	2.190
2	307	1.893	2.227
3	214	1.878	1.963

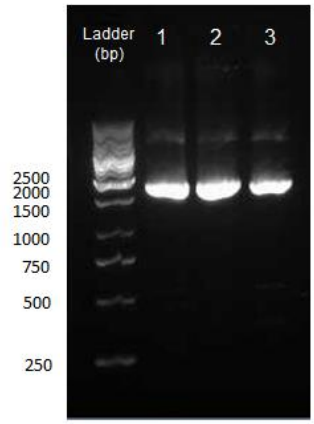

Figure 5. Purified entry clone.

LR reaction

The LR reaction was successfully conducted. The positive control had colonies and the negative one no colonies, simultaneously as colonies appeared onto the sample LB plates. Five colonies of these were picked for further cultivation and three of these resulting cultivations were decided to be purified, since two of the tubes were transparent, indicating no bacterial growth.

The results from the plasmid purification of the final clone, pET-DEST42-*VfLbB*, are presented in table 7 and its corresponding gel in figure 6. The concentrations and ratios were lower,

compared to the ones after the BP reaction. Furthermore, sample three did not have entire plasmids. Sample two, containing pET-DEST42-*VfLbB*, were sent for sequencing and was confirmed to be correct, along with sample two, among the entry clone samples. The colonies from this transformation were used in the cultivation experiments.

Table 7. Final clone, pET-DEST42, VfLbB, concentration measurements and ratios. Sample two from table 6 was used for LR reaction.

Sample	[DNA] ng/µl	A260/A280	A260/A230
1	42.2	1.977	1.889
2	82.0	1.919	1.774
3	54.2	1.912	1.677

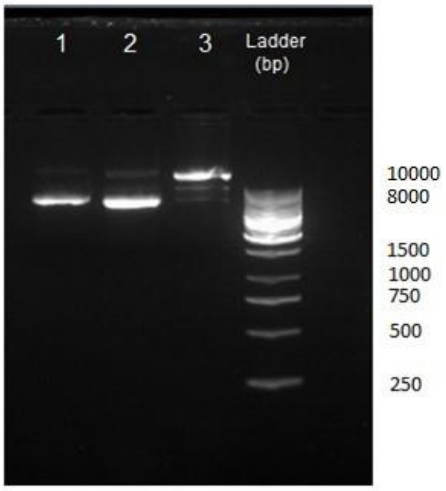

Figure 6. Purified pET-DEST42 VfLbB.

Shake flask experiments

Growth curve of *E. coli* with pET-DEST42-*VfLbB*

The bacterial growth curve for the *E. coli* strain BL21-DE3, containing pET-DEST42-*VfLbB*, is presented in figure 7 and its corresponding logarithmic curve in figure 8. The error bars towards the end of the non-logarithmic curve are more prominent, compared to the rest to the curve. One of the three flasks possessed deviating values and a possible reason for this could be contamination. Another reason could be that the cells produced different levels of inhibiting metabolites which affected their growth (Larsson, 2013).

In the logarithmic curve the deviations are normalized and has less prominent error bars. The specific growth rate and the generation time were calculated to be 1.37 h^{-1} and 30.3 minutes, using the formulas presented in appendix 6. These values are plausible. The generation time of *E. coli* is ranging from 20 to 40 minutes (Expression Technologies Inc., 2003). It is also important to note that the transformed *E. coli* cells in this study also carry a plasmid, which may cause extra maintenance work and hence probably lower growth rate (Larsson, 2013).

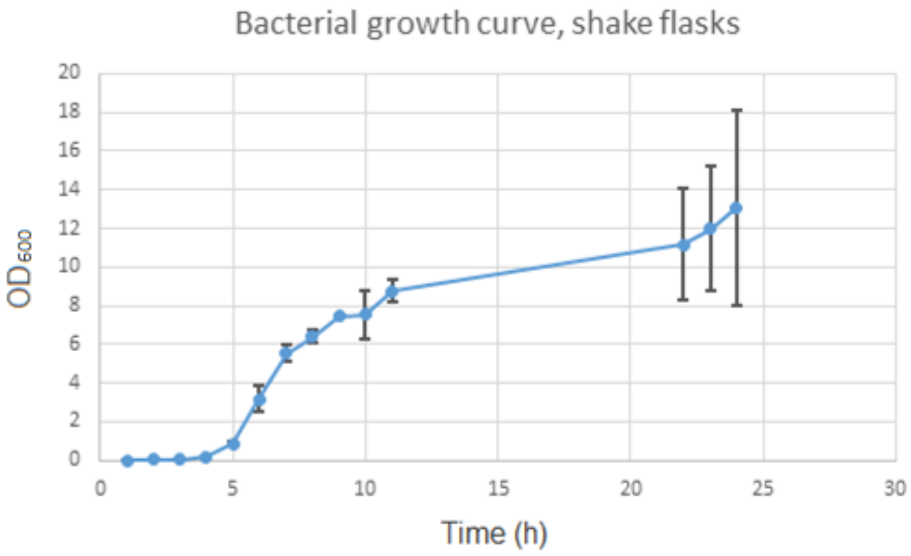

Figure 7. Bacterial growth curve in shake flasks

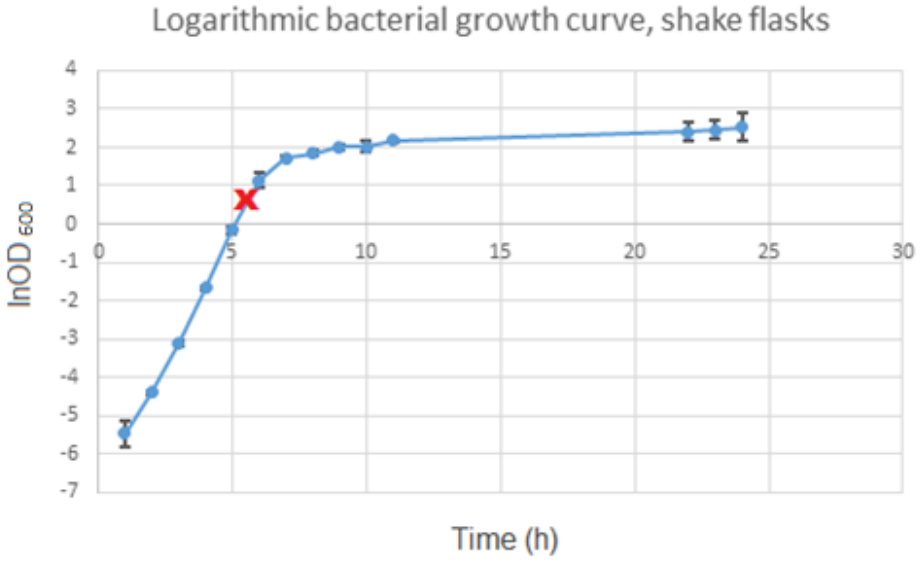

Figure 8. Logarithmic bacterial growth curve in shake flasks. The red cross shows the optimized induction OD_{600}.

VfLbB expression: initial optimization

The VfLbB content in this optimization was compared using AbsV/W, as described on section VfLbB expression analysis in Material and Methods. It was concluded that a stirring rate of 150 was better than 80. No major conclusions regarding CO addition could be drawn, see appendix 2-3 for additional data and spectra (Table 12-22 and Fig.16). Due to the fact that the cell growth is continued after induction, it is plausible that more agitation is better than less when producing VfLbB. The bacteria get better access to the media and thus the metabolic exchange is enhanced (Expression Technologies Inc., 2003).

Time of Induction optimization

Previous research for expressing Lbs in *E. coli* BL21-DE3 has e.g. shown induction OD_{600} at 0.6 and 1.0 when producing soybean Lbs (Hoy & Hargrove, 2008) (Prytulla, et al., 1996). Another example is Lb from *L. luteus* with an induction OD_{600} at 0.5 (Sikorski, et al., 1995). Due to the span of the reported induction OD_{600} values, it was plausible to investigate values which were higher and lower than these.

The results from the optimization are presented in table 8. CO by induction to the flasks in the OD_{600} group 2.0 gave highest mg LbB/gr. An OD_{600} value between 1.5 and 2.0 is in the end of the exponential growth phase of the constructed growth curve (Fig. 8). Other proteins have been expressed at this stage as well, e.g. Larentis et al. (2014) successfully produced *Leptospira* Immunoglobulin-like protein B (LigB) at an OD_{600} value of 2.0 in shake flasks.

Table 8. Time of Induction optimization, see appendix 2-3 for additional data and spectra (Tables 23-30 and Fig. 17).

Optimization condition		**OD_{600} by induction**	**mg LbB/gr**
Induction at 0.0 hours	CO by induction	0.01 ± 0.00	0.020
	CO by harvesting	0.00 ± 0.00	0.118
OD_{600} = 1	CO by induction	1.01 ± 0.05	0.254
	CO by harvesting	0.97 ± 0.06	0.135
OD_{600} = 2	CO by induction	1.87 ± 0.03	0.295
	CO by harvesting	1.80 ± 0.09	0.195

IPTG concentration optimization

From the two previous optimizations, the optimal stirring speed for oxygen diffusion was found as well as the induction OD_{600}, and the time CO bubbling. Among crucial expression variables, the optimization of IPTG and Ala concentrations were the ones left. Previous studies, when

expressing Lbs in E. coli BL21-DE3, have shown induction with final IPTG concentrations of e.g. 0.5 mM for *L. luteus* Lb (Sikorski, et al., 1995) as well as 1.0 mM for soybean Lb (Kundu, et al., 2004) (Hargrove, et al., 1997) (Prytulla, et al., 1996). Four IPTG concentrations were then investigated: 0.1, 0.3, 0.6 and 0.9. The IPTG concentration that gave the highest mg LbB/gr value, see table 9, value was 0.3 mM.

This value is slightly lower than previously produced Lbs, e.g. 0.5 mM for soybean Lb (Kundu, et al., 2004). Although the T7-promoter is IPTG inducible it does not automatically mean the higher concentration the better. Studies have shown that an excessive use of IPTG may cause negative effects to *E. coli*, e.g. slower growth rate and it may also be toxic. Furthermore, lower final IPTG concentrations have been proven to be sufficiently enough to conduct successful protein expression at late induction, i.e. close to stationary phase (Larentis, et al., 2014). The optimized IPTG concentration of 0.3 mM is thus plausible.

Table 9. IPTG concentration optimization, see appendix 2-3 for additional data and spectra (Tables 31-36 and Fig. 18).

Optimization condition	**OD_{600} by induction**	**mg LbB/gr**
[IPTG] = 0.1 mM	1.54 ± 0.05	0.178
[IPTG] = 0.3 mM	1.61 ± 0.06	0.361
[IPTG] = 0.6 mM	1.67 ± 0.10	0.247
[IPTG] = 0.9 mM	1.73 ± 0.25	0.292

Ala concentration optimization

Previous research of Lbs expressed in BL21-DE3 have not used Ala for Lb's expression (Hargrove, et al., 1997) (Kundu, et al., 2004) (Sikorski, et al., 1995) (Prytulla, et al., 1996). On the other hand, Ala has been used in the production of other plant hemoglobins (Leiva Eriksson, 2014) and recombinant human fetal hemoglobin (Ratanasopa, 2015). Thus, it was plausible to evaluate different Ala concentrations. The optimization showed that a final Ala concentration of 0.9 mM gave the highest mg LbB/gr values and hence, according to this study, the VfLbB production is dependent on Ala concentration, see table 10. This result is reasonable since Ala is involved in the synthesis of heme and can be depleted (Ratanasopa, 2015).

Table 10. Ala concentration optimization, see appendix 2-3 for additional data and spectra. (Tables 37-42 and Fig. 19).

Optimization condition	OD_{600} by induction	mg LbB/gr
[Ala] = 0.1 mM	1.53 ± 0.02	0.170
[Ala] = 0.3 mM	1.61 ± 0.07	0.204
[Ala] = 0.6 mM	1.59 ± 0.05	0.188
[Ala] = 0.9 mM	1.56 ± 0.07	0.247

Optimized conditions

After the optimization experiments, the final conditions for expressing VfLbB are: adding CO by induction, induction OD_{600} in the interval 1.5-2.0, a rotation speed of 150 and final IPTG and Ala concentrations of 0.3 as well as 0.9 mM.

The cells obtained in the six flasks (4L) in both of the cultivations run with the finally optimized conditions were merged together. As a result, one tube of cells was obtained for each cultivation. The weights were 16.415 grams in the first run and 25.759 grams in the second. The latter was decided to be further purified and the former was frozen in liquid nitrogen and stored at -80°C for future analysis. The total cell mass in the two optimized shake flask runs differs, i.e. the second run had more than 50% cells than the first. A reason for this could e.g. be that the bacteria in the first run had a shorter stationary phase than the second and/or that they reached this phase at a lower OD_{600} value.

Fermenter

Bacterial growth curve of *E. coli* with pET-DEST42

The bacterial growth curve and its logarithmic equivalent are presented in figure 9 and 10. The actual curve, i.e. the one with OD_{600} vs. time, seems to have some deviating points, compared to the characteristic growth pattern. There are several possible reasons for that. A reason for this could be that OD_{600} measurements were not taken every hour throughout the whole curve length. Another reason could be that the oxygen sensor did not work and hence the stirring rate could not be automated. Thus, optimal oxygen levels were probably not supplied to the bacteria throughout the whole fermentation. Furthermore, between hour 25 and 26, there was a significant increase in pH and during this phase bacterial growth were most likely halted, see appendix 4 for detailed parameter values.

The logarithmic growth curve, which takes the errors into account, looks more plausible, in comparison to the non-logarithmic one. The logarithmic growth curve from the fermenter (Fig. 10) was compared to the curve generated from shake flasks (Fig. 8), in order to convert the optimized induction OD_{600} value to an equivalent one. This value was decided to be 30. The specific growth rate and the generation time were calculated to 0.32 h^{-1} and 2.14 hours. Compared to the values obtained in the shake flasks (Section Growth curve of *E. coli* with pET-DEST42-*VfLbB*), the specific growth rate is lower and the generation time longer. This is plausible since e.g. the temperature in the shaking flasks was 37ºC and in the fermenter it was 30ºC, the latter is not optimal for cell growth. Furthermore, parameters such as the media differed: TB media was used in the shaking flasks and DM in the fermenter (Expression Technologies Inc., 2003) .

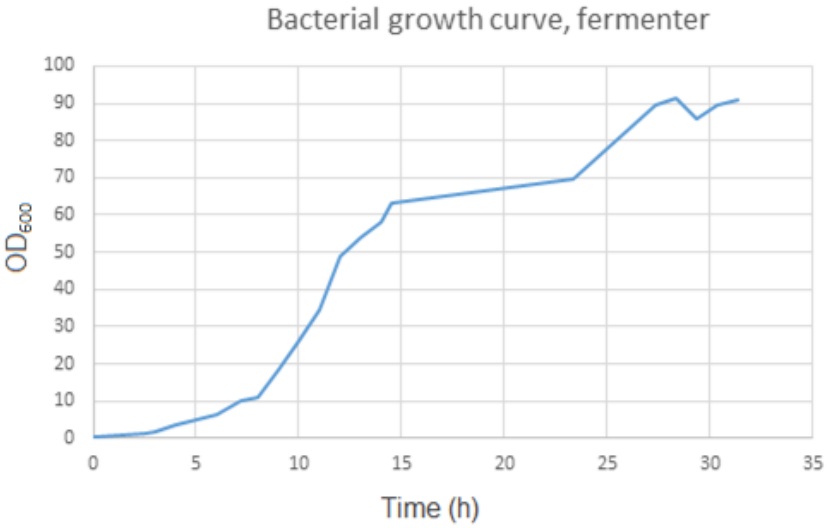

Figure 9. Bacterial growth curve in fermenter.

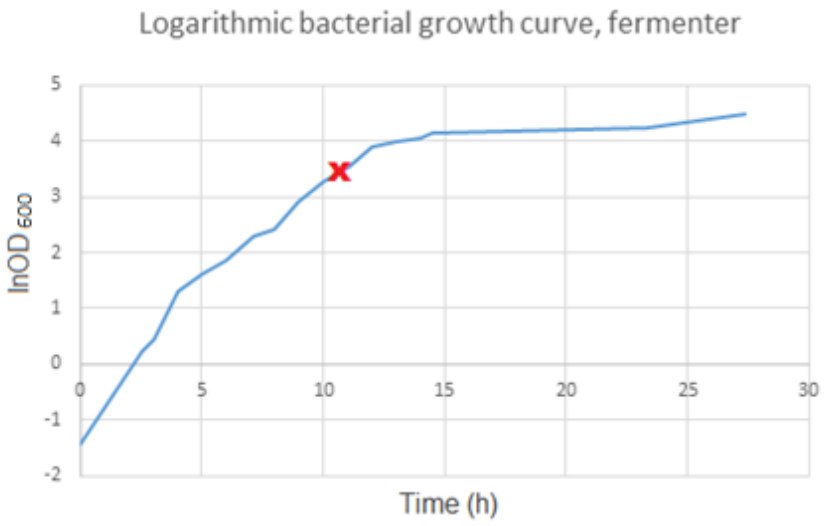

Figure 10. Logarithmic bacterial growth curve in fermenter. The red cross in the figure shows the converted, optimized induction OD_{600}.

VfLbB expression

The cell concentration in the harvested volumes were 110 ± 6 grams per liter. The values from these duplicates were similar and thus, from these experiments, it can be concluded that the different induction OD_{600} values do not affect the total cell concentrations.

Dynamics interpretation

In figure 11-13, continuously registered fermentation parameters are presented for the three runs. As stated before, the oxygen sensor did not work during the first run and this parameter is thus absent in figure 11. This can be related to the stirring pattern, since the stirrer is automated to a fixed pO_2 value. As a result, the stirring in the second and third run (Fig. 12 and 13) was fluctuating more than the first run. Regarding the pO_2 curves in the second and third run, they are at a constant pace during the first hours, indicating cell growth. By induction, the O_2 set-point was manually decreased to 2.5%, which can also be observed in the figures. The glucose concentration was manually measured throughout the fermentations. Once the glucose was depleted the fed-batch mode was started, where after the introduced glucose volume increased over time. In all of the three fermentations, the base automatically increased over time as a result of a pH set-point. It is plausible that the base volume increases gradually and this due to the bacterial production of acidic overflow metabolites, e.g. lactic acid and acetic acid, and hence a

decrease in pH (Larsson, 2013).

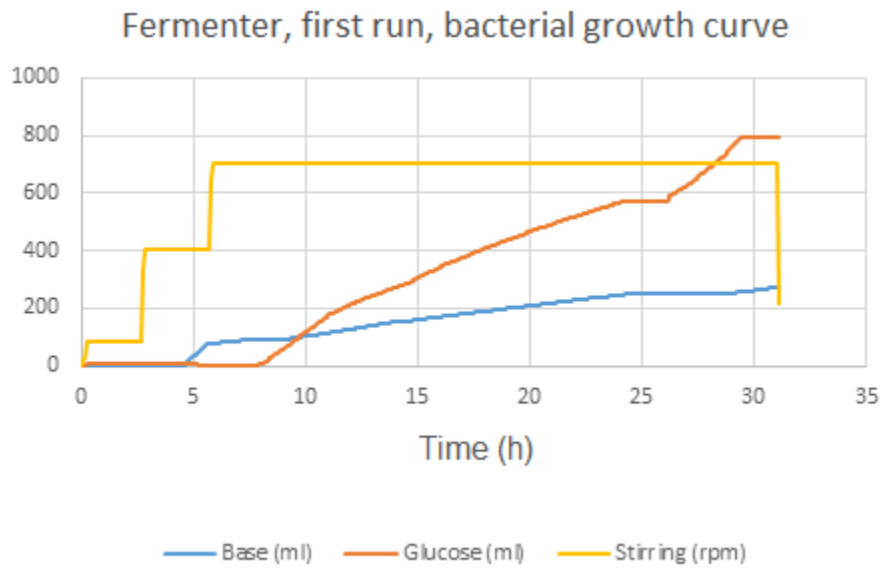

Figure 11. Fermentation parameters, first run.

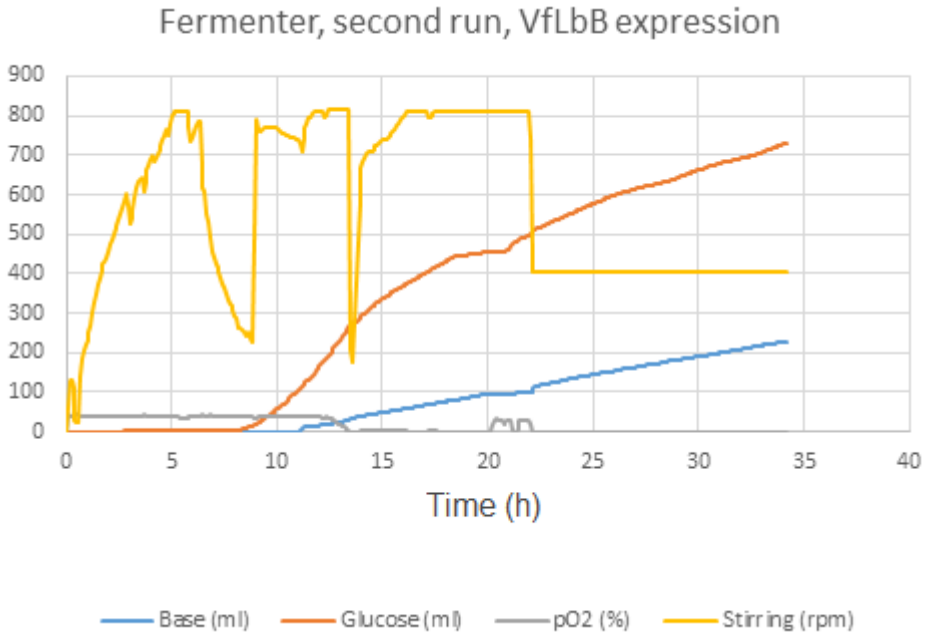

Figure 12. Fermentation parameters, second run.

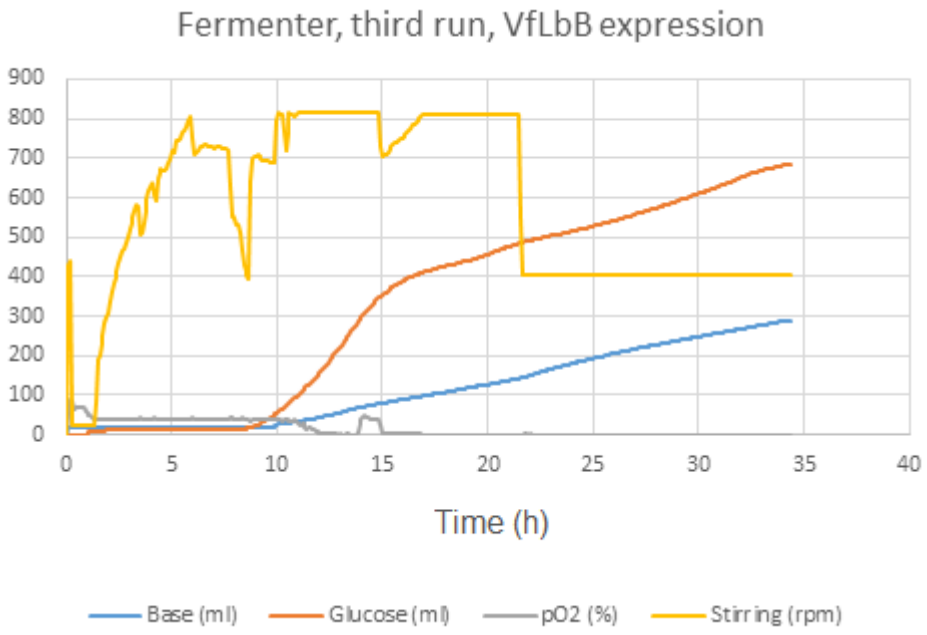

Figure 13. Fermentation parameters, third run.

CO essay

In figure 14, a spectra of VfLbB after dialysis and sterile filtration is shown. After NaD and CO were added to the samples, the spectra shifted to the right. Hardgrove et al. (1997) demonstrated this with soybean Lb. The absorbance maxima for soybean Lb in the oxy-state was 409 and 416 in the CO-state. VfLbB has a smaller shift to the right. Its Fe(II)O_2 peak is located at 415 and its Fe(II)CO peak at 417.

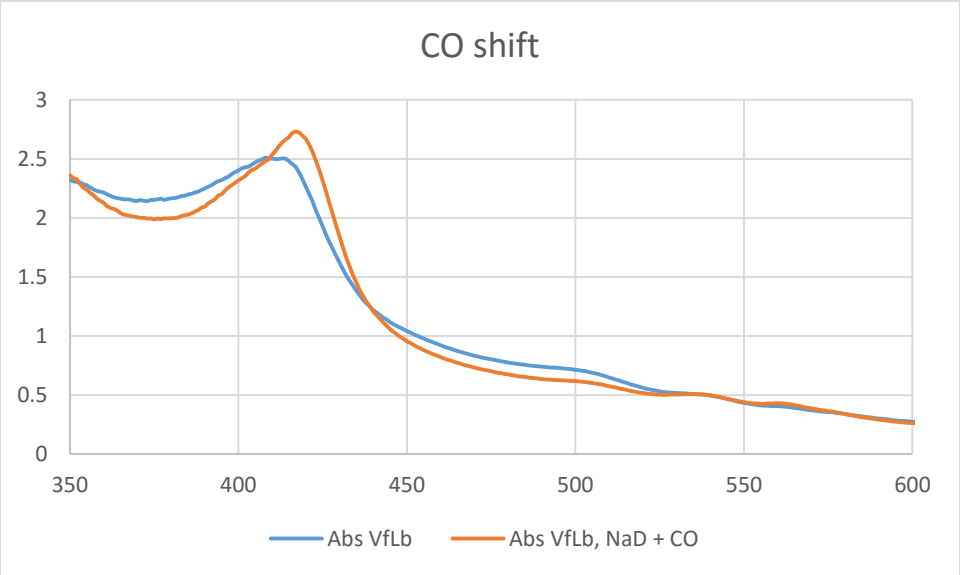

Figure 14. CO shift, peak characterization.

Purification results

In table 11, the mg LbB/gr values are presented for the dialysed and filtered samples. The second fermentation run with induction OD_{600} at 30.0 showed the highest mg LbB/gr after dialysis, independently of the buffer used (NaP or Tris-HCl). Theoretically, the shaking flasks should have given the same LbB/gr values as the second fermentation run, since this fermentation was based on the optimized conditions from the shaking flasks, e.g. the final IPTG concentration and time of induction OD_{600}.

The difference in LbB/gr between the second fermentation run and the shaking flasks is just a minor one. They are in the same range. However, a reason for this small deviation, that shaking flasks produce less, could be that six individual samples of cell pellets were merged together into one tube. Although these shake flask samples had the same conditions, one or two deviating ones may affect the average.

Moreover, the third fermentation gave the lowest mg LbB/gr values compared to the second fermentation (Table 11) and the only difference between them was the induction OD_{600}. A concluding remark from this is that the bacteria should rather be induced at OD_{600} 30, instead of 50, in order to obtain as high Lb concentration as possible. As stated before, an OD_{600} value of 30 is in the end of the exponential growth phase of the growth curve. Thus, a value of 50 is at the very beginning of the stationary phase or in the interphase of the two. The exponential growth phase seems to have an influence in protein production in this case.

Table 11. Dialyzed and filtered samples, Tris-HCl and NaP buffers. The spectra are available in Fig. 20-21, Appendix 3.

Sample	mg LbB/gr
Shake flasks (NaP buffer)	0.227
Shake flasks (Tris buffer)	0.264
Fermentation 2 (NaP buffer)	0.266
Fermentation 2 (Tris buffer)	0.296
Fermentation 3 (NaP buffer)	0.179
Fermentation 3 (Tris buffer)	0.236

In figure 15, the results from the QFF column for the shake flask sample is presented. VfLbB, represented by a blue line in the chromatogram, did not bind the column successfully. Other proteins, detected at 280nm and represented by a purple line, did bind better on the other hand. This was also the result for the QFF of the second fermentation sample and for both the samples analyzed in the CaptoS exchange chromatography experiments, see the other chromatograms in appendix 5 (Figs. 22-25).

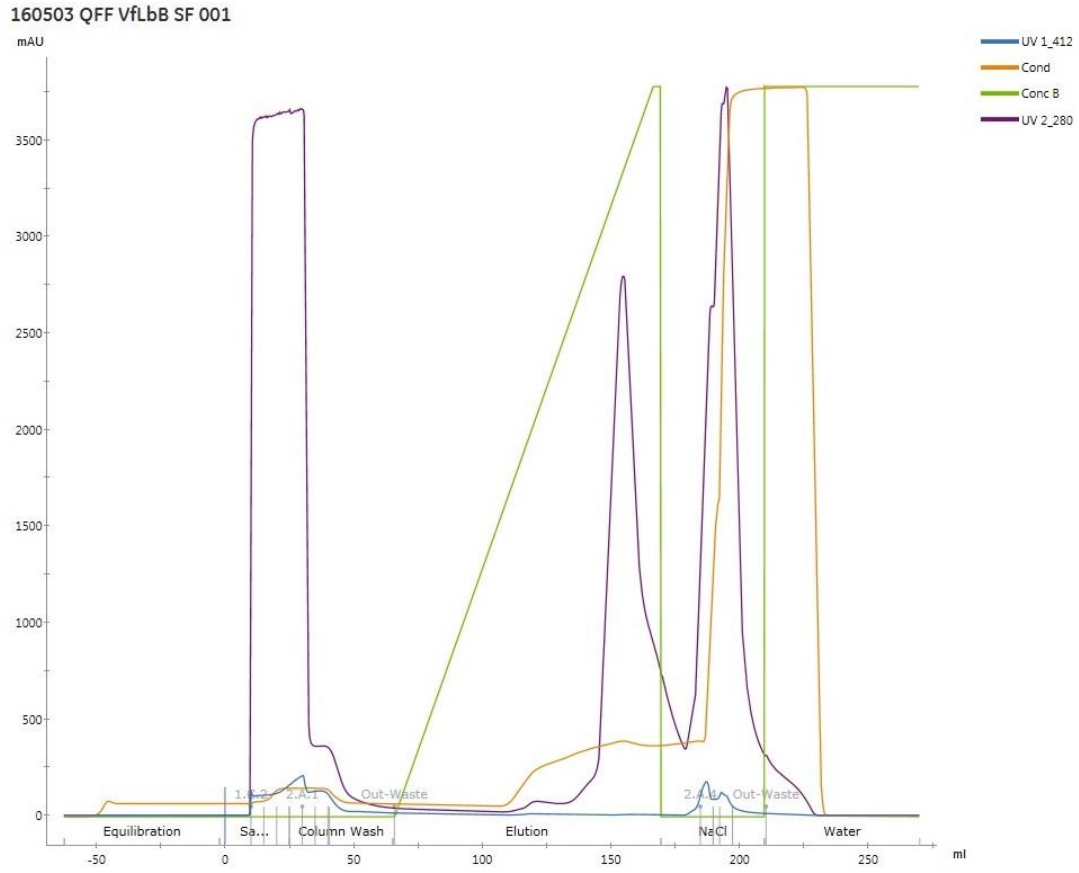

Figure 15. QFF, VfLbB from shake flasks.

It was unexpected that VfLbB neither bound to the CaptoS nor the QFF. The pI value of VfLbB is, as stated before, 7.7 (Frühling, et al., 1997) and both the ion-exchange buffers were designed according to that. A possible reason why the VfLbB did not bind any of the columns could be that a component in the buffers interfere with the protein to some extent or that the column interfere with some parts of the three dimensional structure of VfLbB. Arredondo-Peter et al. (1997) used a purification procedure consisting of several steps, when purifying recombinant

Cowpea Lb II. This purification included precipitation with ammonium sulphate, chromatography and electrophoresis. Regarding the chromatography methodology in this study, both ion-exchange and hydrophobic variants were used.

Future research

In this project, gene cloning, shake flask optimizations, production in a fermenter and initial purification were conducted. Due to time constraints, neither optimization of the fermenter conditions, e.g. glucose feeding profile, oxygen concentration, pH, temperature, nor characterization experiments, except from CO peak shift characterization, of VfLbB could be carried out. Thus, this is a suggestion for future research.

Regarding the future characterization, kinetics studies of VfLbB would be plausible to conduct. When parameters such as the oxygen association and dissociation rates have been determined, further modifications of the VfLbB molecule, e.g. PEGylation, should be conducted, in order to evaluate if this could be a blood substitute candidate. The pI value of VfLbB is 7.7, which is close to the one of Hb in human blood, is a promising characteristics.

Furthermore, optimization in the ion-exchange purification process, e.g. in a similar systematic manner as for the induction parameters in the shaking flasks, could also be another thing to consider. Another suggestion is conduct affinity exchange chromatography, as an extension to ion-exchange chromatography, and also using ammonium sulphate precipitation. Protein engineering techniques could also be applied to the VfLbB gene, in order to obtain other desirable characteristics of the protein molecule.

Eventually, determine an extinction coefficient for VfLbB would be plausible as future research. In this thesis the extinction coefficient for *L. luteus* Lb was used and an extinction coefficient would enable calculations of correct concentrations.

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Appendix 1

Machines and equipment

Centrifugation of 1.5 and 2.0 ml Eppendorf tubes:

Spectrafuge 24D (Labnet International Inc)

Centrifugation of 50 ml tubes:

SORVALL® RC 6 PLUS (Thermo Electron Corporation), rotor: FIBERLite F13-14x50cy (Thermo Scientific)

Centrifugation of 250 ml tubes:

SORVALL® RC 6 PLUS (Thermo Electron Corporation), rotor: FIBERLite F14-6x250 (Piramoon Technologies Inc)

SORVALL RC-5B Refrigerated Superspeed (DuPont Instruments), rotor: SORVALL SLA-1500 SUPER-LITE

Sonicator:

QSONICA SONICATORS, 20 kHz output frequency

Incubators:

Innova™ 4430 Incubator Shaker

Innova® 44 Incubator Shaker Series

Gallenkamp Cooled Orbital Incubator

Heatblocks:

Thermo Block TDB-120 (Biosan)

Thermomixer Compact (Eppendorf)

Heaters and stirrers:

MR 3001 K (Heidolph)

IKAMAG® REO (Drehzahl Electronic)

Fermenter:

BIOSTAT® A Plus, 5 liter vessel + software (Sartorius Stemdim biotech)

Spectrophotometers:

NanoPhotometer (Implen)

Cary 60 UV-Vis (Agilent Technologies)

Electrophoresis:

Equipment from Bio-Rad

Chemicals:

From SigmaAldrich

Cloning kit, Gateway Technology:

From Invitrogen

Chromatography:

ÄKTA, GE Healthcare

HiPrep™ Q FF 16/10 (anion exchange column), GE Healthcare

HiScreen™ Capto™ S (cation exchange column), GE Healthcare

Dialysis:

Spectra/Por®1 Dialysis Membrane. Standard RC Tubing. Molecular cut off weight: 6-8 kD

Plasmid purification kits:

Nucleospin® Gel and PCR Clean-up (Macherey-Nagel-07/2015 Rev.03)

E.Z.N.A. ® Plasmid DNA Mini Kit I

Appendix 2

VfLbB expression: initial optimization

Table 12. OD_{600} = 3.0 ± 0.5,150 rpm, CO by induction.

Flask 660 µl	OD_{600} (Induction)	OD_{600} by harvesting
4	3.46	-
5	2.38	-
6	3.09	-

Table 13. Group 1 – OD_{600} 0.8, 80 rpm, CO by induction.

Flask 250 µl	OD_{600} (Induction)	OD_{600} by harvesting
1	0.710	2.880
2	0.754	1.920

Table 14. Group 2 - OD_{600} 0.8, 80 rpm, CO by harvesting.

Flask 250 µl	OD_{600} (Induction)	OD_{600} by harvesting
3-4	0.93 ± 0.22	3.83 ± 0.23

Table 15. Group 3 – OD_{600} 0.8, 150 rpm, CO by induction.

Flask 250 µl	OD_{600} (Induction)	OD_{600} by harvesting
5-6	1.01 ± 0.28	5.24 ± 1.15

Table 16. Group 4 – OD_{600} 0.8, 150 rpm, CO by harvesting

Flask 250 µl	OD_{600} (Induction)	OD_{600} by harvesting
7-8	0.87 ± 0.08	8.53 ± 1.00

Table 17. Group 5 – OD_{600} 1.5, 80 rpm, CO by induction.

Flask 250 µl	OD_{600} (Induction)	OD_{600} by harvesting
9-10	1.50 ± 0.20	2.71 ± 0.02

Table 18. Group 6 – OD_{600} 1.5, 80 rpm, CO by harvesting.

Flask 250 µl	OD_{600} (Induction)	OD_{600} by harvesting
11-12	1.40 ± 0.21	4.56 ± 0.44

Table 19. Group 7 – OD_{600} 1.5, 150 rpm, CO by induction.

Flask 250 µl	OD_{600} (Induction)	OD_{600} by harvesting
13-14	1.36 ± 0.07	6.02 ± 0.20

Table 20. Group 8 – OD_{600} 1.5, 150 rpm, CO by harvesting.

Flask 250 µl	OD_{600} (Induction)	OD_{600} by harvesting
15-16	1.51 ± 0.10	8.06 ± 0.19

Table 21. VfLbB expression: initial optimization, real absorbance, volumes, weight.

Sample	Real absorbance	Lysate Vol (ml)	Cell weight (gr)	Abs*V	AbsV/W
1	0.15	5.5	1.05	0.81	0.77
2	0.81	2.0	0.69	1.22	2.37
3-4	0.73	5.5	2.53	4.04	1.60
5-6	0.85	8.5	3.64	7.22	1.98
7-8	1.02	12.5	5.00	12.74	2.55
9-10	0.29	3.5	1.79	1.01	0.57
11-12	0.92	6.0	2.59	5.51	2.13
13-14	0.87	10.0	4.26	8.71	2.05
15-16	0.78	12.5	4.92	9.75	1.98
1*	0.94	11.5	5.06	10.82	2.14
2*	0.78	11.0	4.99	8.56	2.32
3*	0.61	12.5	5.61	7.65	2.32

- 1*, 2* and 3* were the tubes, obtained from the 2L shake flasks cultivation, the rest are from one liter shake flasks.

Table 22. Conclusion VfLbB expression: initial optimization.

Sample	rpm	CO	AbsV/W
7-8	150	By harvesting	2.55
2	80	By induction	2.37
3*	150	By induction	2.32
2*	150	By induction	2.32

1*	150	By induction	2.14
11-12	80	By harvesting	2.13
13-14	80	By induction	2.05
5-6	150	By induction	1.98
15-16	150	By harvesting	1.98
3-4	80	By harvesting	1.60
1	80	By induction	0.77
9-10	80	By induction	0.57

Time of Induction optimization

Table 23. Group 1 – immediate induction, CO by induction.

Flask 250 µl	**OD_{600} (Induction)**	**OD_{600} by harvesting**
13-14-15	0.01 ± 0.00	1.53 ± 0.01

Table 24. Group 2 - immediate induction, CO by harvesting.

Flask 250 µl	**OD_{600} (Induction)**	**OD_{600} by harvesting**
17-18-19	0.00 ± 0.00	3.11 ± 0.24

Table 25. Group 3 - OD_{600} 1, CO by induction.

Flask 250 µl	**OD_{600} (Induction)**	**OD_{600} by harvesting**
2-3-4	1.01 ± 0.05	5.81 ± 2.15

Table 26. Group 4 - OD_{600} 1, CO by harvesting.

Flask 250 µl	**OD_{600} (Induction)**	**OD_{600} by harvesting**
6-7-8	0.97 ± 0.06	9.57 ± 1.17

Table 27. Group 5 - OD_{600} 2, CO by induction.

Flask 250 µl	**OD_{600} (Induction)**	**OD_{600} by harvesting**
10-11-12	1.87 ± 0.03	4.44 ± 1.17

Table 28. Group 6 - OD_{600} 2, CO by harvesting.

Flask 250 µl	OD_{600} (Induction)	OD_{600} by harvesting
1-5-9	1.80 ± 0.09	9.80 ± 1.84

Table 29. Time of Induction optimization, real absorbance, volumes, weights.

Sample	OD_{600} (Induction)	CO	Real absorbance	Lysate Vol (ml)	Cell weight (gr)	mg LbB/gr
1-5-9	2	By harvesting	1.01	16.0	6.68	0.195
2-3-4	1	By induction	1.30	9.5	4.05	0.254
6-7-8	1	By harvesting	0.68	18.5	7.98	0.135
10-11-12	2	By induction	1.57	9.0	3.98	0.295
13-14-15	Immediate	By induction	0.11	4.0	1.80	0.020
17-18-19	Immediate	By harvesting	0.61	7.5	3.23	0.118

Table 30. Time of Induction optimization, conclusion.

Sample	OD_{600} (Induction)	CO	mg LbB/gr
10-11-12	2	By induction	0.295
2-3-4	1	By induction	0.254
1-5-9	2	By harvesting	0.195
6-7-8	1	By harvesting	0.135
17-18-19	Immediate	By harvesting	0.118
13-14-15	Immediate	By induction	0.020

IPTG concentration optimization

Table 31. Group 1 - 0.1 mM final IPTG concentration.

Flask 250 µl	OD_{600} (Induction)	OD_{600} by harvesting
1-2-3	1.54 ± 0.05	4.81 ± 0.23

Table 32. Group 2 - 0.3 mM final IPTG concentration.

Flask 250 µl	OD_{600} (Induction)	OD_{600} by harvesting

4-5-6	1.61 ± 0.06	4.44 ± 0.13

Table 33. Group 3 - 0.6 mM final IPTG concentration.

Flask 250 µl	OD_{600} (Induction)	OD_{600} by harvesting
7-8-9	1.67 ± 0.10	4.65 ± 1.41

Table 34. Group 4 - 0.9 mM final IPTG concentration.

Flask 250 µl	OD_{600} (Induction)	OD_{600} by harvesting
10-11-12	1.73 ± 0.25	4.77 ± 0.05

Table 35. IPTG concentration optimization, real absorbance, volumes, weights.

Sample	IPTG concentration (mM)	Real absorbance	Lysate Vol (ml)	Cell weight (gr)	mg LbB/gr
1-2-3	0.1	0.98	8.5	3.90	0.178
4-5-6	0.3	1.91	9.5	4.18	0.361
7-8-9	0.6	1.40	7.5	3.53	0.247
10-11-12	0.9	1.54	9.0	3.95	0.292

Table 36. IPTG concentration optimization, conclusion.

Sample	IPTG concentration (mM)	mg LbB/gr
4-5-6	0.3	0.361
10-11-12	0.9	0.292
7-8-9	0.6	0.247
1-2-3	0.1	0.178

Ala concentration optimization

Table 37. Group 1 - 0.1 mM final Ala concentration.

Flask 250 µl	OD_{600} (Induction)	OD_{600} by harvesting
1-2-3	1.53 ± 0.02	2.63 ± 0.33

Table 38. Group 2 - 0.3 mM final Ala concentration.

Flask 250 µl	OD_{600} (Induction)	OD_{600} by harvesting
4-5-6	1.61 ± 0.07	3.49 ± 0.16

Table 39. Group 3 - 0.6 mM final Ala concentration.

Flask 250 µl	OD_{600} (Induction)	OD_{600} by harvesting
7-8-9	1.59 ± 0.05	2.56 ± 0.27

Table 40. Group 4 – 0.9 mM final Ala concentration.

Flask 250 µl	OD_{600} (Induction)	OD_{600} by harvesting
10-11-13	1.56 ± 0.07	3.49 ± 0.21

Table 41. Ala concentration optimization, real absorbance, volumes, weights.

Sample	Ala concentration (mM)	Real absorbance	Lysate Vol (ml)	Cell weight (gr)	mg LbB/gr
1-2-3	0.1	0.95	6.5	3.02	0.170
4-5-6	0.3	1.21	7.0	3.46	0.204
7-8-9	0.6	1.03	7.0	3.19	0.188
10-11-13	0.9	1.47	7.0	3.47	0.247

Table 42. Ala concentration optmization, conclusion.

Sample	Ala concentration (mM)	mg LbB/gr
10-11-13	0.9	0.247
4-5-6	0.3	0.204
7-8-9	0.6	0.188
1-2-3	0.1	0.170

Optimized conditions

Table 43. Optimized shake flask conditions, round 1.

Flask 660 µl	OD_{600} (Induction)	OD_{600} by harvesting
1-2-3-4-5-6	1.61 ± 0.09	2.52 ± 0.03

Table 44. Optimized shake flask conditions, round 2.

Flask 660 µl	**OD_{600} (Induction)**	**OD_{600} by harvesting**
1-2-3-4-5-6	1.56 ± 0.07	5.81 ± 0.81

Table 45. Weights, pellet merged flasks, optimized conditions.

Sample	**Weight (g)**
1-2-3-4-5-6 (first run)	16.42
1-2-3-4-5-6 (second run)	25.76

Appendix 3
VfLbB spectra

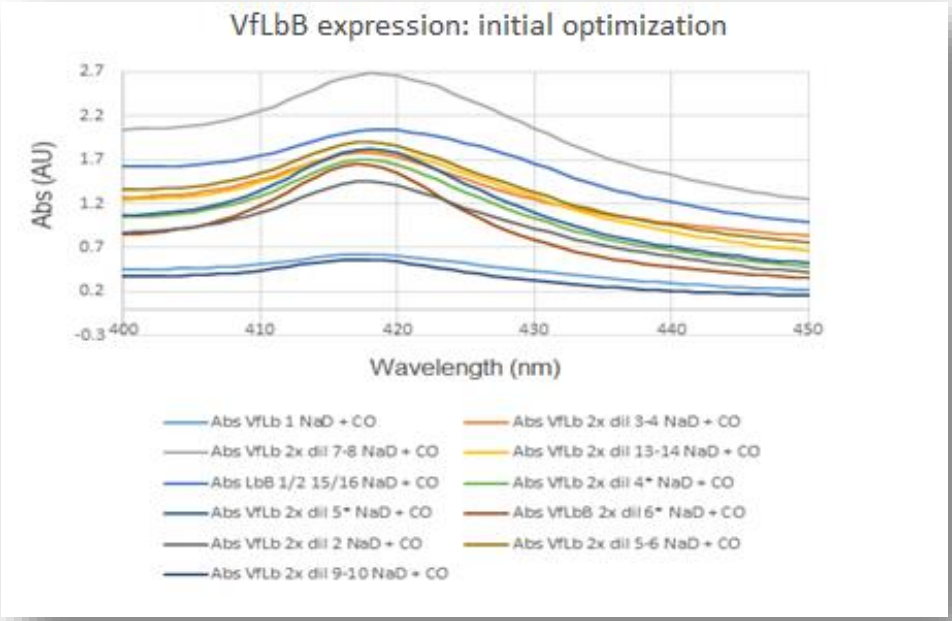

Figure 16. Spectra, VfLbB expression: initial optimization.

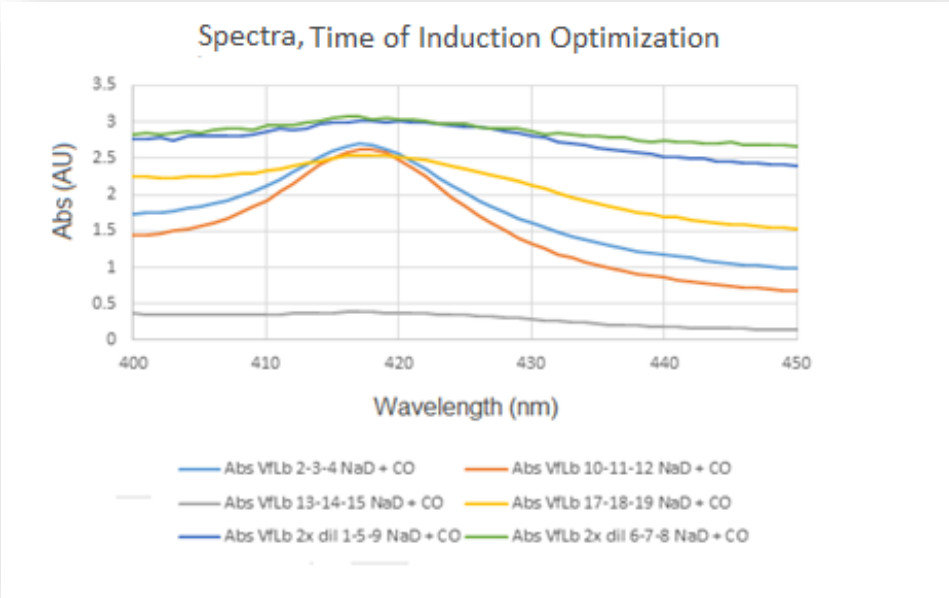

Figure 17. Spectra, OD_{600} by induction and CO addition optimization.

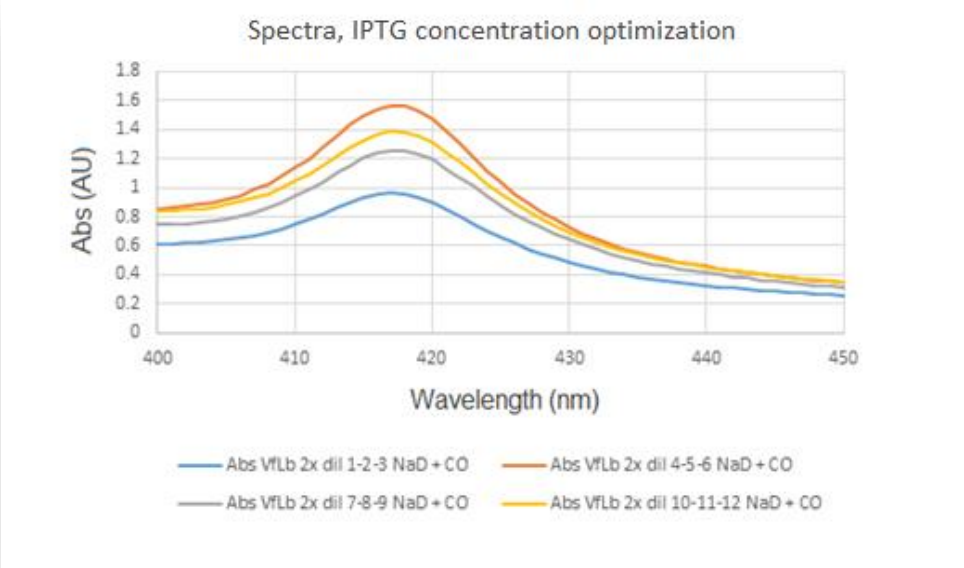

Figure 18. IPTG concentration optimization.

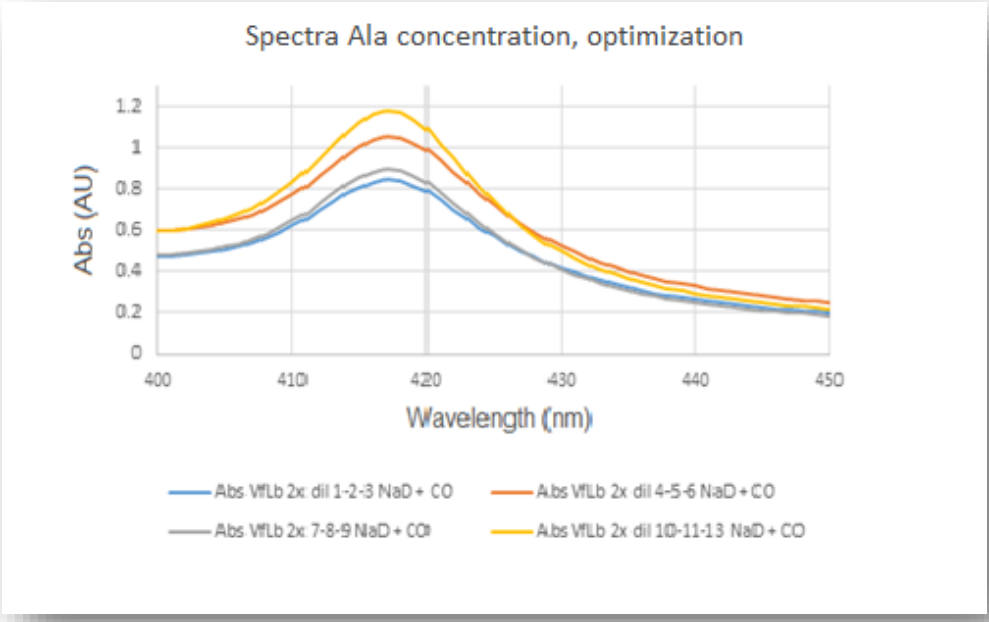

Figure 19. Ala concentration optimization.

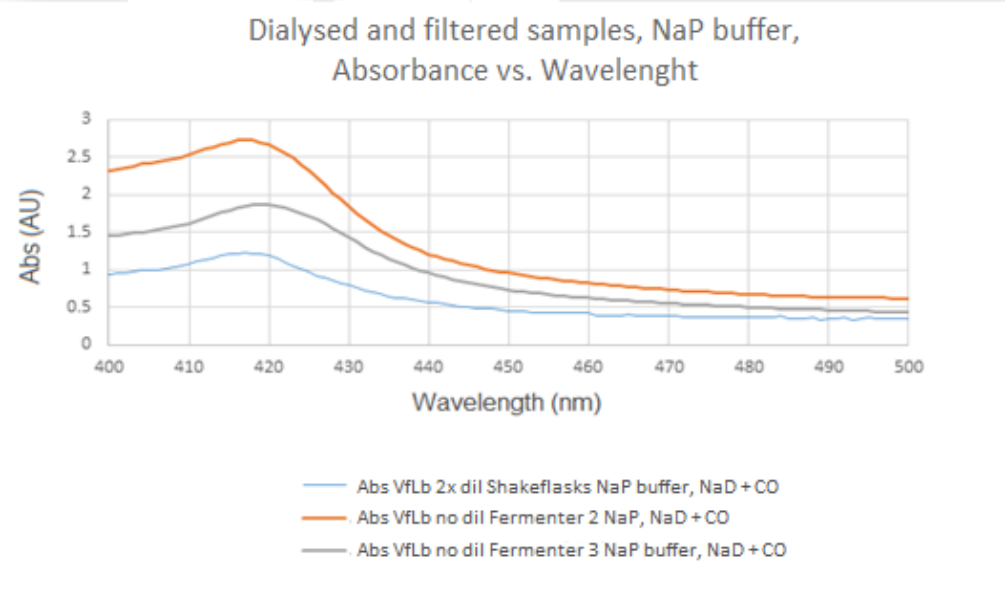

Figure 20. Dialysed and filtered samples, NaP buffer.

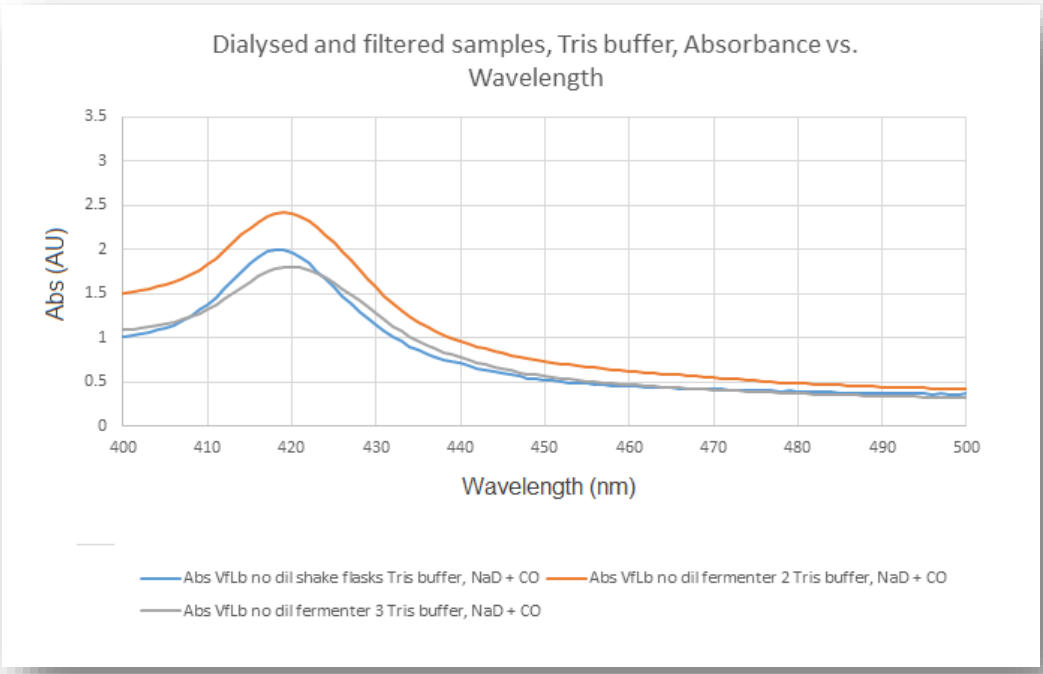

Figure 21. Dialysed and filtered samples, Tris buffer.

Appendix 4

Fermentation data

Fermentation 1

Hours	OD_{600}	pH	base	glu (ml)	glu (mM)	profile (%)	air (1 bar)	stirr	pO2 (%)	cells (mg/ml)
0	0.237	7.4	0	0	52	0	5	21	-	
2.5	1.262	7.17	0	0	44	0	5	87	-	4.8
3	1.551	7.01	0	0	43	0	7	406	-	5
4	3.72	6.86	0	0	37	0	7	406	-	7.6
5	5.08	6.64	12	0	27	0	7	406	-	9.6
6	6.5	6.71	78	0	17.8	0	7	703	-	9.2
7.1667	9.88	6.7	87	0	4.2	0	7	703	-	15
8	11.2	6.86	90	3	-	1.5	9	704	-	16.7
9	18.2	6.71	90	45	21.7	5.2	9	704	-	26.8
10	26.3	6.7	101	102	43	5.7	9	704	-	36.6
11	34.6	6.7	112	160	71	5.5	9	704	-	46.5
12	49	6.7	124	204	62	3.4	9	704	-	53.7
13	53.8	6.7	137	237	41	2.8	9	704	-	63.9
14	58.2	6.7	147	260	16	2.6	9	704	-	69.4
14.5	63.1	6.7	155	278	11.1	2.5	9	704	-	71.1
23.33	69.5	6.7	238	545	-	2.2	9	704	-	84.6
24.33	80	6.7	246	565	-	2.1	9	704	-	85.9
27.33	89.8	6.98	248	629	17.3	5	9	704	-	95
28.33	91.6	6.61	248	688	41	6.2	9	704	-	98.2
29.33	86	6.51	254	763	91	7.4	8	704	-	97
30.33	89.5	6.5	263	792	103	7.1 (off)	8	704	-	107.4
31.33	90.8	6.5	272	792	99.9	5.3 (off)	8	704	-	92.8

- The fermenter was started at 8:10 on April 20 and stopped at 15:30
- O_2 sensor not behaving, max O_2: 474%
- At 3 hours, the stirring was increased to 405 rpm and airflow to 7 liters per minute
- At 6 hours, the stirring was increased to 700 rpm.
- The profile was started at 8.5 hours, due to low glucose levels
- At 8 hours, the airflow was increased to 9 liters per minute.
- At 10 hours the profile was changed to 5% 2h, 3% 4h, 2%, 6h, 1% 14h
- At 11 hours the profile was changed to 4% 0h, 3% 1h, 2% 5h, 1% 8h
- At 15 hours, the profile was changed to 4% 0h, 3% 2h, 2% 10h, 1% 12h
- Something might interfere with the glucose: 1/10 and dilution showed “low” on the glucose device and ½ dilution as well as no dilution showed “E-3”, which means either extremely high value or device/strip error.

- At 23 hours, it was noticeable that the base had accumulated in the pellet.
- The glucose pump was turned off at 24.5 hours.
- The glucose pump was started at 26.5 hours
- The glucose pump was stopped at 29.5 hours, due to high glucose value.

Fermentation 2

hours	OD_{600}	pH	base	glu (ml)	glu (mM)	profile	air (1 bar)	stirr	pO2 (%)	cells (mg/ml)
0	0.595	7.23	0	0		0	5	21	40.5	
11	13.9	6.37	9	115	26.4	5	10	750	39.7	29.7
12	21.8	6.51	16	167	21.8	6	10	810	37.4	31.5
13	29.2	6.49	25	236	17.4	7	10	814	14.9	45.3
15.5	44.7	6.5	53	355	17.1	3	8	740	2.2	66.6
17.5	61.3	6.49	72	419	19.5	2.8	8	810	1.5	91.7
19.5	88.6	6.5	91	450	28.6	2.6	8	810	0	126.4
21.5	94.1	6.78	97	487	low	2.4	8	810	32.7	142.8
34	98	6.5	229	730	high	1.7	8	405	0	136.1

- pO_2 check, 80.2% (11 L min and 800 rpm)
- The fermenter was started at 20:30 on April 25 and stopped at 06:30 on April 27.
- The pH meter was recalibrated to 7.55 after the first sampling.
- At 11 hours, the airflow was changed from 5 liters per minute to 10 liters per minute.
- The pH was switched on (pH meter was accidentally turned off) and set to 6.5 and auto.
- At 13.5 hours, the airflow was changed to 8 liters per minute.
- At 18.5 hours, the glucose pump was turned off (profile on)
- At 19 hours, the pump was switched on again (profile still on)
- At 19 hours (5 minutes after previous), the pump was switched off again due to high glucose value.
- At 20.5 hours, the pump was switched on due to low glucose levels and high pH (7.15)
- At 21 hours, 10 ml glucose was added manually, thereafter auto.
- At 22 hours, 5 ml glucose was added manually
- At 22 hours, the stirring was changed from 100% MAX to 30%MAX (810 rpm to approx. 405)
- The induction was conducted at approximately OD_{600} 30, at 13.5 hours. The IPTG concentration was 0.3 mM and Ala 4 mM. Dark conditions, 22^{o}C and O_2 set-point of 2.5%.
- The harvested volume was measured to 3.6 L

Fermentation 3

hours	OD_{600}	pH	base	glu (ml)	glu (mM)	profile	air (1 bar)	stirr	pO2 (%)	cells (mg/ml)
0	0.386	6.63	0	0		0	5	21	67.5	
11	25.4	6.5	39	135	32.4	5.3	5	810	17.8	36.9
12	34	6.5	47	173	18.6	5.9	5	815	0.5	44.8
13	46.6	6.5	60	248	19.1	7	10	815	0.1	64.6
15.5	63.6	6.5	87	384	16.3	2.9	8	800	2	77.8
17.5	73.6	6.5	105	424	15.1	1.3	8	810	0	105.7
19.5	76.4	6.5	125	453	14.9	1.7	8	811	0	121.4
21.5	94.5	6.5	142	485	19.6	1.3	8	811	0.1	127
34	96.4	6.5	290	685		1	8	405	0	130.2

- pO_2 check, 91.0%
- The fermenter was started at 20:30 on April 27 and stopped at 06:30 on April 29.
- Small leakage during the night, to volume of approximately 3 liter.
- At 13 hours the airflow was changed to 10 L min.
- The profile before induction was: 8h 0.1%, 9h 3%, 11h 5%, 13h 7%, 15h 8%
- The profile after induction was: 0h 5%, 2h 3%, 10h 2%, 14h 1%, 16h 2%, 18h 1%, 29h 2%, 22h 1%
- At 21.5 hours, the stirring max was changed to 30% (from 800 to 400 rpm).
- The induction was conducted at approximately OD_{600} 50, at 13.5 hours. The IPTG concentration was 0.3 mM and Ala 4 mM. Dark conditions, 22°C and O_2 set-point of 2.5%.
- Due to the spill, the harvested volume was measured to 3.1 L

Table 46. Total grams of cells and concentration of cells per liter, fermentation 2 and 3

Sample	Fermentation 2 Weight (g)	Fermentation 3 Weight (g)
1	33.12	18.72
2	44.32	30.881
3	22.62	23.629
4	32.063	33.033
5	39.56	31.88
6	28.344	30.202
7	26.893	31.355
8	29.42	30.433
9	24.656	26.812
10	37.023	38.53
11	37.851	31.399
12	28.912	
13	27.22	
Total weight (g)	412.002	326.874
g/L	114.445	105.4432

Appendix 5

Chromatograms

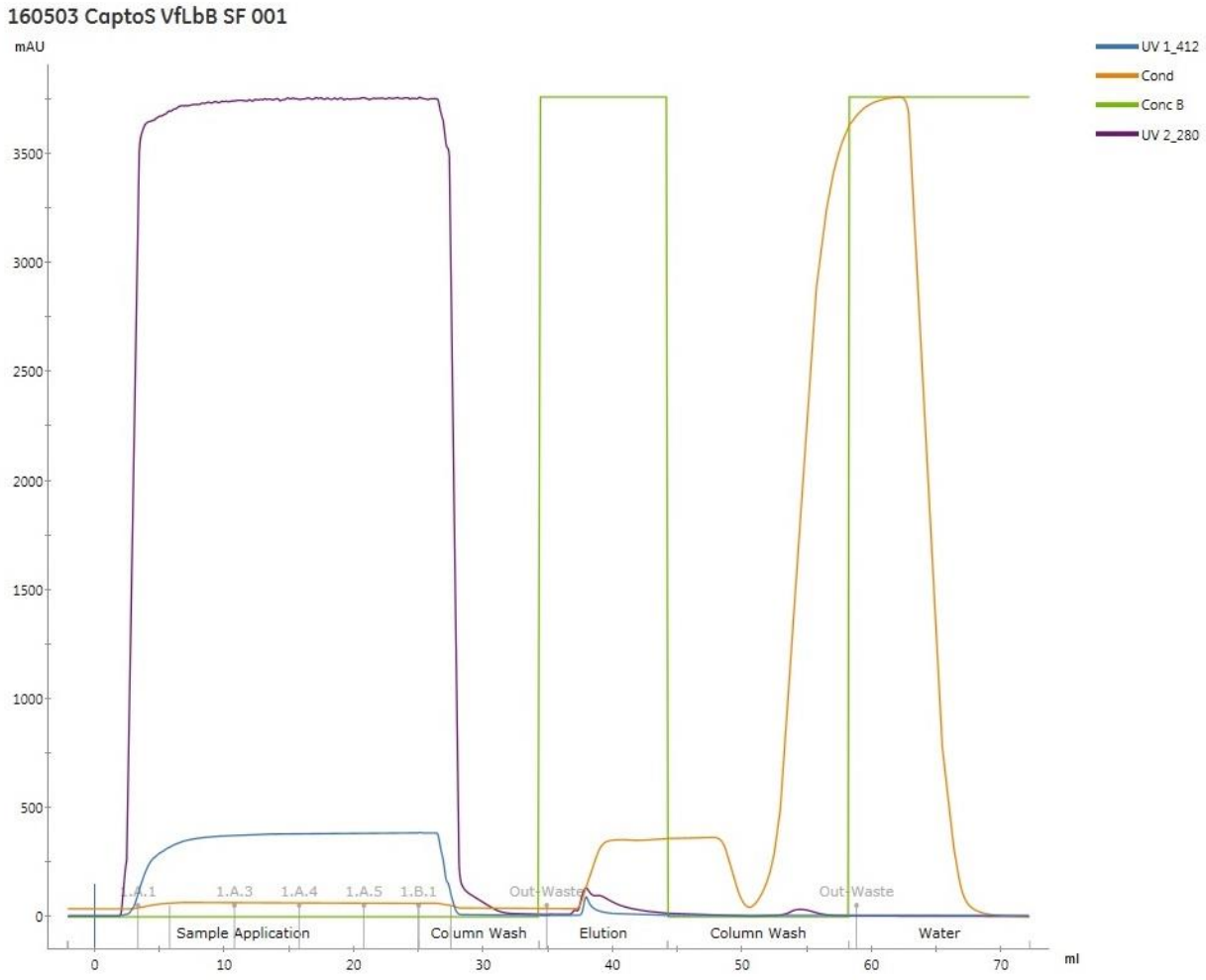

Figure 22. CaptoS VfLbB, shake flask cultivation chromatogram.

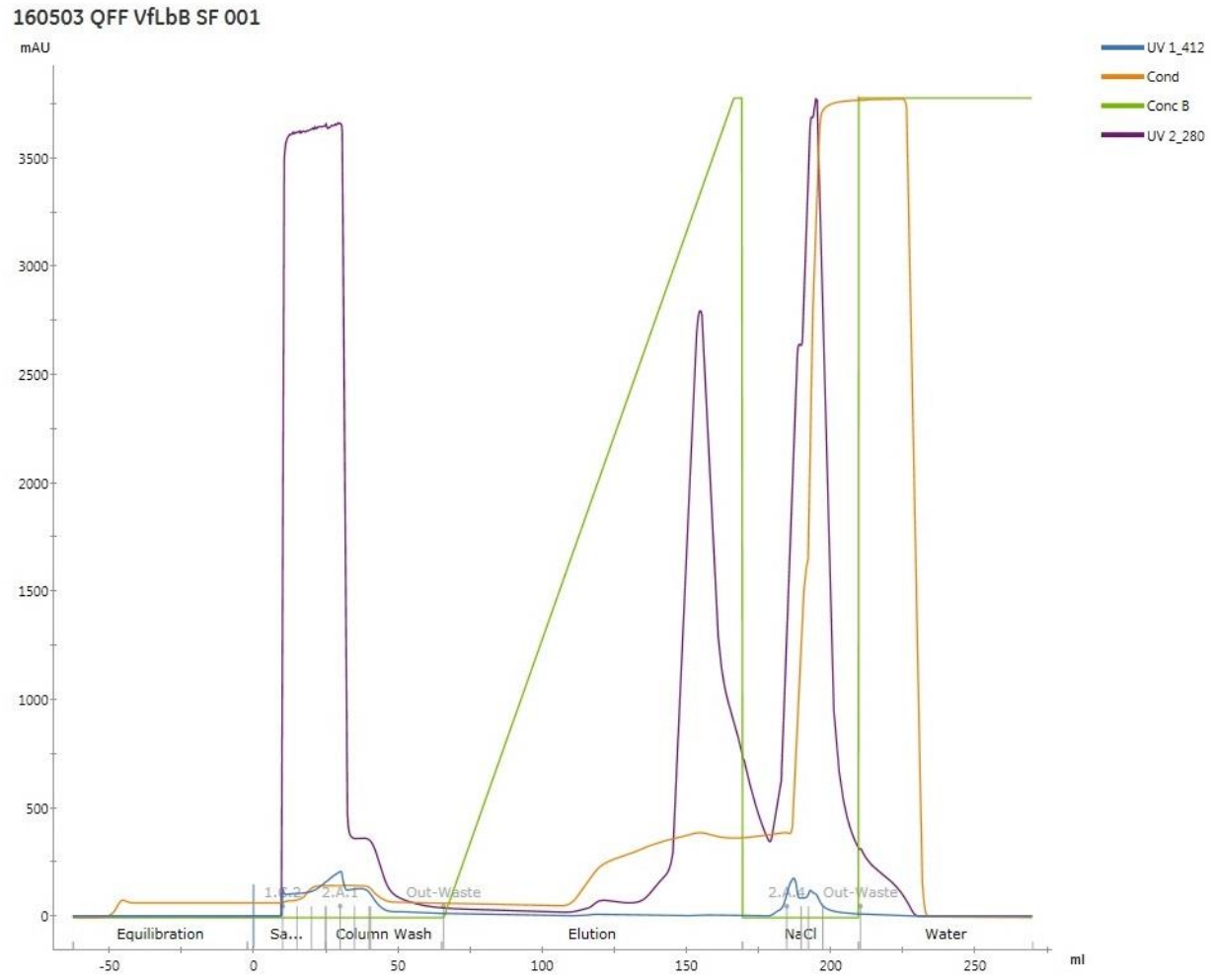

Figure 23. QFF VfLbB, shake flask cultivation chromatogram.

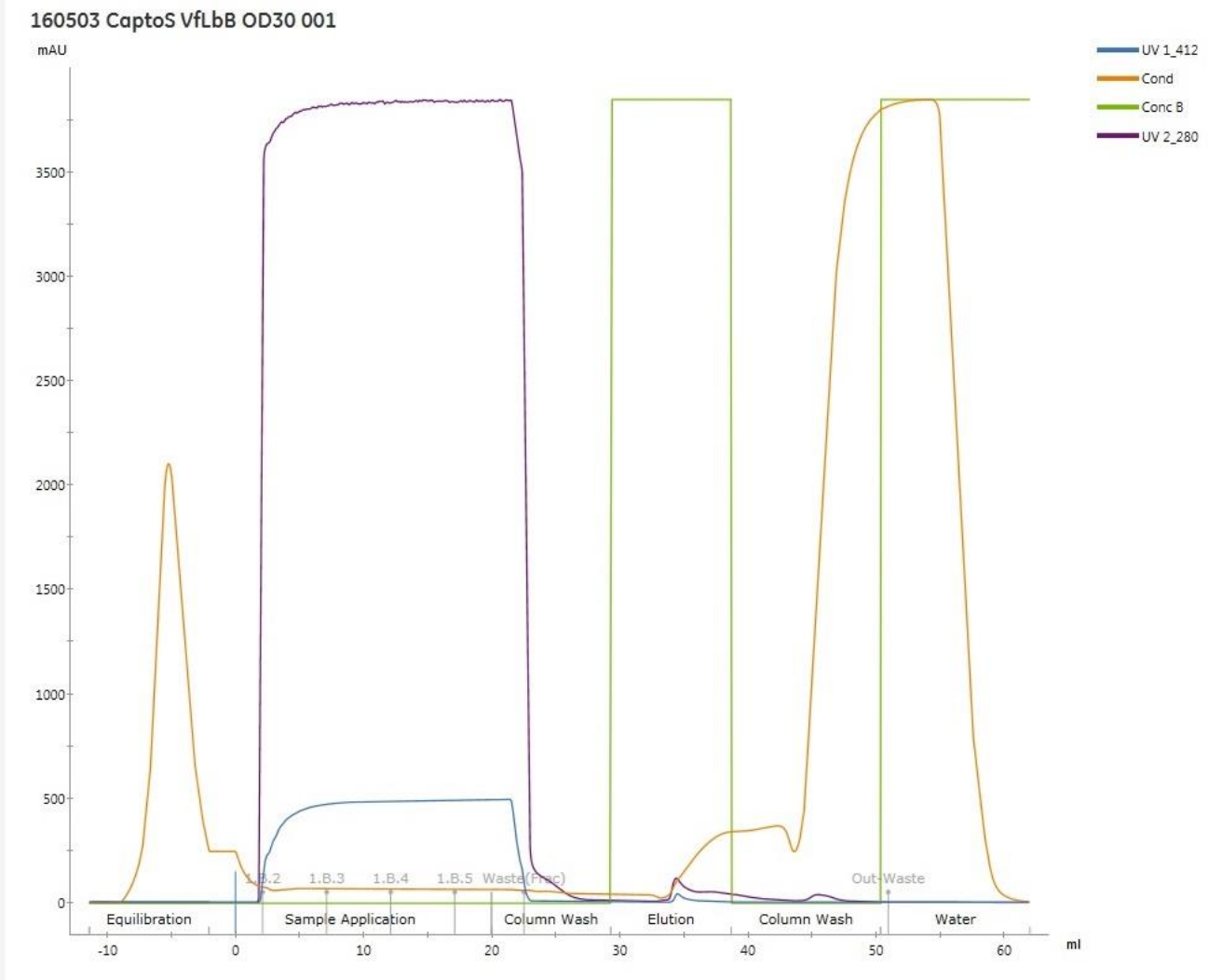

Figure 24. CaptoS VfLbB fermentation, OD_{600} 30, chromatogram.

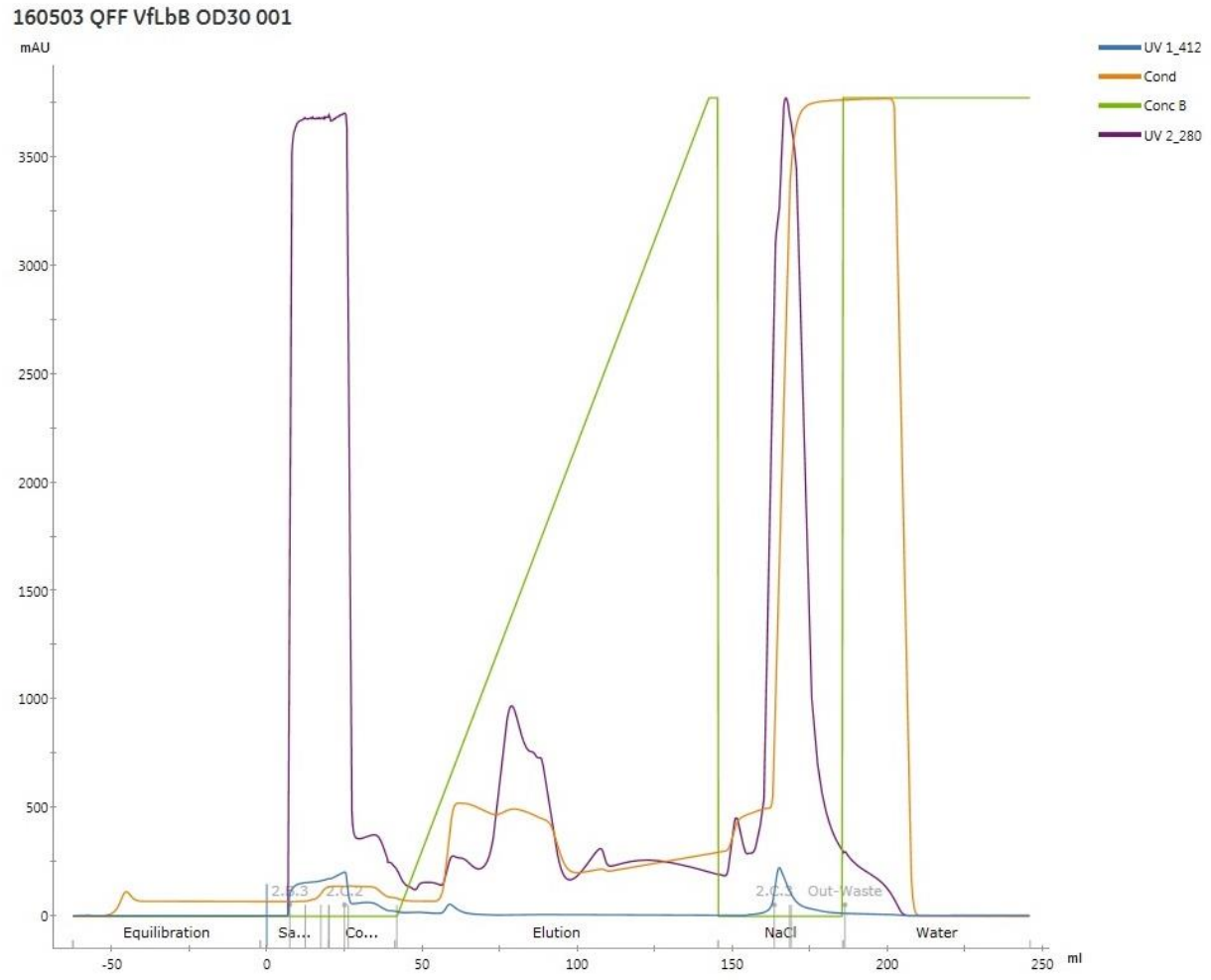

Figure 25. QFF VfLbB fermentation, OD_{600} 30, chromatogram.

Appendix 6

Microbial kinetics calculations:

The following formulas were used to calculate the specific growth rate, μ, and the generation time, t_g:

$$\mu = \frac{\ln \frac{x_2}{x_1}}{t_2 - t_1}$$

where letter x stands for cell mass, e.g. OD_{600}, and t for time. The numbers 1 and 2 are indices.

$$t_g = \frac{\ln 2}{\mu}$$

(Larsson, 2013)