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Process development for the control of solubility of Affibody® molecules

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Both target proteins are of therapeutic interest. One of the proteins is an IL2 fusion protein (Z03358-ABD094-(S4G)3-IL2) that bind the platelet-derived growth factor receptor β (PDGFR-β). PDGF signaling is of interest in cancer treatment where, among other things, the effects of PDGF on tumor angiogenesis is researched. The His6-(Z05477)2 protein has a classified target but is developed as a therapeutic in the area of inflammation and autoimmune disease. Both model proteins are known to be difficult to purify due to low solubility.

The two *E. coli* expression systems investigated and compared were BL21(DE3) and Lemo21(DE3). The fusion protein Z03358-ABD094-(S4G)3-IL2 was produced in BL21(DE3) in inclusion bodies with a yield of 4.95 g/l. An optimized process for the expression of His6-(Z05477)2 using BL21(DE3) was developed with a yield of 6.6 g/l soluble protein after expression at 30°C for 6 h.

Keywords; Protein expression, Platelet derived growth factor receptor-β (PDGFR- β), Expression systems, High cell density cultivations (HCDC).

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Process development for the control of solubility of Affibody® molecules

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Populärvetenskapling sammanfattning

Detta examensarbete handlar om att producera biologiska läkemedel på ett så säkert och kostnadseffektivt sätt som möjligt. Det finns ett stort behov av nya målsökande proteinläkemedel mot bland annat cancer och autoimmuna sjukdomar. Antikroppar är ett modernt biologiskt läkemedel som ofta används för att målsöka och inhibera olika cancermarkörer.

Affibodies är en annan form av målsökande biologiska läkemedel, som detta arbete främst handlar om. Det finns ett antal svårigheter med att hitta nya protein läkemedel där kostnaden och eventuella immunologiska reaktioner är två av de främsta. Det är otroligt dyrt att utveckla och producera nya läkemedel samt att de ofta påverkar kroppen på oönskade sätt som gör att bara ett fåtal potentiella läkemedel godkänns. Under arbetets gång har fokus legat på att på ett så effektivt sätt som möjligt producera två utvalda proteiner som eventuellt kommer användas som biologiska läkemedel en dag.

Under arbetets gång har ett flertal tekniker för proteinframställning använts, som high cell density cultivations (HCDC), för maximal protein produktion. Ett antal vanliga analysmetoder för att bedöma renhet och produktionsmängd har utnyttjats. De två proteiner som arbetet handlar om är tänkt att ha olika effekt i människokroppen. Båda proteinerna är tänkta att trigga immunförsvaret och på så sätt hjälpa kroppen att bekämpa sjukdom, det ena mot cancer och det andra mot inflammation och autoimmunitet.

Examensarbete 30 hp Civilingenjörsprogrammet Molekylär bioteknik

Uppsala Universitet, Oktober 2011

Abstract

In this study the aim was to optimize the production of the Affibody fusion-protein Z03358-ABD094-(S4G)3-IL2 with regard to the amount of soluble protein produced. However, problems with reproducibility with this protein and the chosen expression system were encountered. Therefore, expression of the His-tagged Affibody His6-(Z05477)2 was evaluated using the same expression system as well as expression in another well characterized expression system.

Both target proteins are of therapeutic interest. One of the proteins is an IL2 fusion protein (Z03358-ABD094-(S4G)3-IL2) that binds the platelet-derived growth factor receptor β (PDGFR-β). PDGF signaling is of interest in cancer treatment where, among other things, the effects of PDGF on tumor angiogenesis are studied. The His6-(Z05477)2 protein has a classified target but is developed as a therapeutic in the area of inflammation and autoimmune disease. Both model proteins are known to be difficult to purify due to low solubility.

The two *E. coli* expression systems investigated and compared were BL21(DE3) and Lemo21(DE3). Response surface methodology was used for optimization of soluble expression of the two proteins. The parameters varied were L-rhamnose concentration, temperature, time of induction, expression time and glucose feed rate. Expression was performed using small-scale batch cultivations in different formats, as well as large-scale high cell density cultivations (HCDC). A quantification method using densitometry was developed. The optimization protocol development was hampered by the fact that it proved difficult to achieve reproducible results with Lemo21(DE3). The fusion protein Z03358-ABD094-(S4G)3-IL2 was produced in BL21(DE3) in inclusion bodies with a yield of 4.95 g/l. An optimized process for the expression of His6-(Z05477)2 using BL21(DE3) was developed with a yield of 6.6 g/l soluble protein after expression at 30°C for 6 h.

Table of Contents

1 Background

1.1 Introduction

In the area of biopharmaceuticals Monoclonal antibodies (MAbs) are to date the most successful biomedicine. The reason for this is the specificity with which they bind their target, as well as the ability to create MAbs that binds almost any possible target[1]. In recent years several disadvantages with using MAbs for this purpose have surfaced. The main disadvantages are their large size, complicated composition and the issue of intellectual property.

1.1.1 Affibody® molecules

Affibody® molecules are a class of small proteins (6.5kDa) based on the B-domain of the immunoglobulin-binding region of *staphylococcal* protein A[1]. The B-domain consists of 58 amino acids that are folded into a three-helical bundle and after mutating key positions for enhanced chemical stability the engineered variant is called the Z-domain[2]. An Affibody® molecule consists of a non-cysteine three-helix bundle domain and can be selected to bind a large number of targets. The binding properties are varied through genetic variation of 13 randomized positions on the surface of the Z-protein scaffold[3], (Fig. 1).

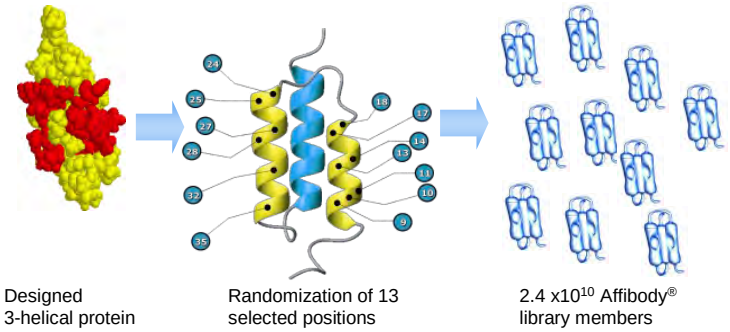


Figure 1. Schematic image of the randomization of 13 amino acid positions. A library of binders are created with up to 2.4x10^{10} Affibody® molecules and the best binders in regard to affinity are chosen for maturation.

There are a number of advantages with using Affibody® molecules. It is a small single-chain protein and this makes most selection technologies applicable as well as making the construction of fusion proteins possible[1]. Fast folding, general high solubility and a relatively high thermal stability make these molecules interesting for development of therapeutics as well as diagnostics in the form of imaging agents. Another advantage with Affibody® molecules is the possibility of production by chemical peptide synthesis. This can simplify the production process of molecules with incorporated chemical moieties like chelating groups or fluorescent probes[2]. The main advantage with chemical peptide synthesis is a reduced risk of contaminating, biologically active molecules, leading to lesser regulatory demands on chemically synthesized proteins in comparison with biologically produced proteins.

a  b 

Figure 2. Image of the three-helix bundle domain of an Affibody® Molecule (a) and the Albumin Binding Domain from streptococcal protein G (b).

Affibody® AB has developed a unique Albumin binding technology, Albumod™ that extends the circulatory half-life of biopharmaceuticals that can be coupled to both Affibody® molecules as well as other potential pharmaceuticals[4]. Figure 2 illustrates the general structure of both an Affibody® molecule (Fig. 2a) and an Albumin binding domain (ABD, Fig. 2b). Human serum albumin (HSA) has an average half-life of 19 days. Several properties like lack of toxicity and immunogenicity make it an ideal candidate for drug delivery[5]. ABD bind HSA with very high affinity and due to the very tight binding a fusion protein containing ABD can gain a circulatory half-life identical to serum albumin[4]. Because of their bacterial origin Affibody® molecules can be efficiently expressed in prokaryotic expression systems like *E. coli,* whereas full size antibodies require production in eukaryotic expression systems[6].

1.1.2 Expression systems

Escherichia coli is the most commonly used host for recombinant protein production partly because it is so far the best characterized expression system[7]. Transformation of *E. coli* with foreign DNA is relatively easy with well-established genetic manipulation methods, and this is a major advantage when using this expression system for protein production. This means that it is possible to create stable, over-expressing cell lines in a short time. *E. coli* has a fast growth rate compared to mammalian cells and produce large quantities of protein[8]. When expressing recombinant proteins in *E. coli* there are some common problems. The expression of recombinant protein often results in insoluble and/or nonfunctional protein. Aggregation of over-expressed recombinant proteins can result from accumulation of folding intermediates or insufficient processing by molecular chaperones[9]. *E. coli* lack the ability to perform the post translational modifications like glycosylation, something that certain eukaryotic proteins need for correct folding and this often lead to biologically inactive protein[9]. Recombinant protein that is expressed as inclusion bodies can sometimes be refolded into a soluble and active form, but it can be difficult to regain its biological activity[10]. There are a few strategies in use today for dealing with this problem and one of them is controlling the *E. coli* intracellular milieu. This includes expression at lower temperatures, using genetically modified *E. coli* strains, modification of media composition and co-expression of molecular chaperones[10]. Two *E. coli* strains used in recombinant protein production are BL21(DE3) and Lemo21(DE3).

Bacteriophage T7 RNA polymerase (T7RNAP) is often used to drive recombinant protein production in *E. coli.* In BL21(DE3) and its derivates, the gene encoding the T7RNAP is inserted into the chromosome under control of the *lac*UV5 promoter. The lacUV5 promoter is a strong variant of the wild-type *lac* promoter, insensitive to catabolite repression and therefore only controlled by the *lac* repressor, LacI. When the artificial inducer isopropyl β-D-thiogalactoside (IPTG) is added, the *lac*I repressor is relieved and the result is recombinant protein production[11]. Since some complex proteins are insoluble when using BL21(DE3), other strains have been developed to try and change the expression from insoluble to soluble. One such expression system is Lemo21(DE3) (Fig. 3) that have been developed for expression of proteins that are hard to express in soluble form, such as membrane proteins and other poorly soluble proteins [11]. Lemo21(DE3) is a derivative of *E. coli* BL21(DE3) that has an L-rhamnose based tunable system for protein over-expression[11]. In Lemo21(DE3) the activity of the T7 RNA polymerase can be controlled by its natural inhibitor, the T7 lysozyme (T7Lys)[11]. Expression of T7Lys is in turn controlled by the rhaBAD promoter[12]. The rhaBAD promoter is an L-rhamnose inducible promoter and titrating L-rhamnose may enable soluble protein production[13]. Lemo21(DE3) is a good alternative expression system for production of recombinant proteins because of the possibility to fine-tune the expression, and thereby making the production of soluble protein possible. Figure 3, is a schematic image illustrating the Lemo21(DE3) expression system.

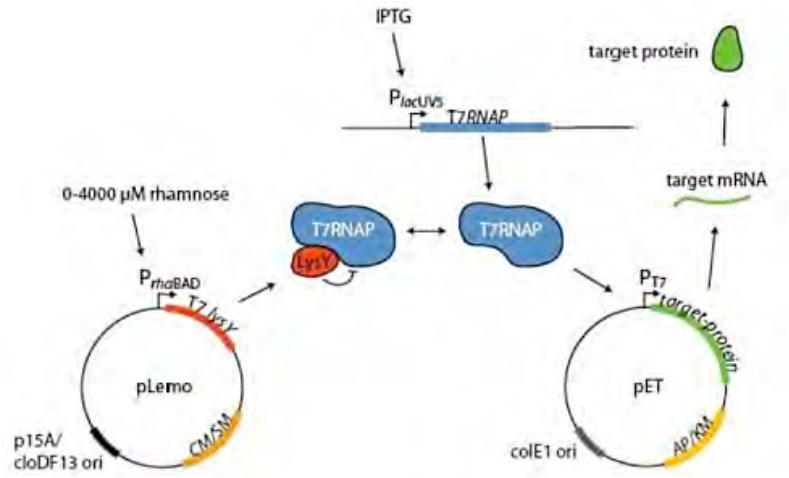


Figure 3. Schematic view of the Lemo21(DE3) system. Adding rhamnose activates the rhoBAD promoter and T7 Lys is transcribed. T7Lys then inhibits the T7RNA polymerase and the expression of target protein is hampered.

1.1.3 Fermentation methods

Two of the most used fermentation methods are batch and fed-batch cultivations. In batch cultivations all nutrients are added from the beginning, and this means limited control of the growth of the culture. This can lead to changes in the growth medium such as changes in pH and substrate depletion. Batch cultivations only result in limited cell densities. Therefore, only limited product formation is obtained when using this method.

In fed-batch cultivation growth can be controlled by limiting the energy source according to the rate of consumption. Fed-batch cultivations are used to reach high cell density cultivations (HCDC). HCDC is used for recombinant protein production since an increase in cell density increases productivity[14].

1.1.4 MODDE

Multivariate data analysis can be used to optimize the fermentation process. MODDE (Umetrics) is a software program that works by using response surface methodology to make predictions. The program help in the design of the experiments with regard to a given question/questions. A common approach is to create a standard reference experiment (center-point) and perform experiments around it[15]. Specific factors can be excluded after the experiments have been conducted if the results and model show that they are not important for the model. The program can give a calculated optimum of the process parameters. The variables varied in this study were L-rhamnose concentration, temperature, time of induction, expression time and glucose feed rate.

1.1.5 Target proteins

Both of the proteins studied in this project are of therapeutic interest. The Affibody® molecule Z03358-ABD094-(S4G)3-IL2 is a PDGFR-β binder that is fused to an albumin binding domain and an IL2 molecule. PDGF signaling has been investigated as a target for cancer treatment due to its effect on growth stimulation of tumor cells as well as its role in tumor angiogenesis[16]. The idea is that it will have both a blocking effect of the PDGFR-β, but most importantly, the IL2 molecule is meant to work as an effector molecule. One strategy of tumor targeting is based on the accumulation of biopharmaceuticals around new blood vessels[17]. Studies support the idea that effective immunotherapy with an IL2 cytokine is achieved through a cytotoxic T-cell mediated response[18]. The second protein, His6-(Z05477)2 has a classified target but is meant to be used as a therapeutic for autoimmune disease and inflammation.

Table 1. The proteins and their molecular weight (MW).

Protein	**MW**
Z03358-ABD094-(S4G)3-IL2	28349.83 Da
His6-(Z05477)2	14494 Da

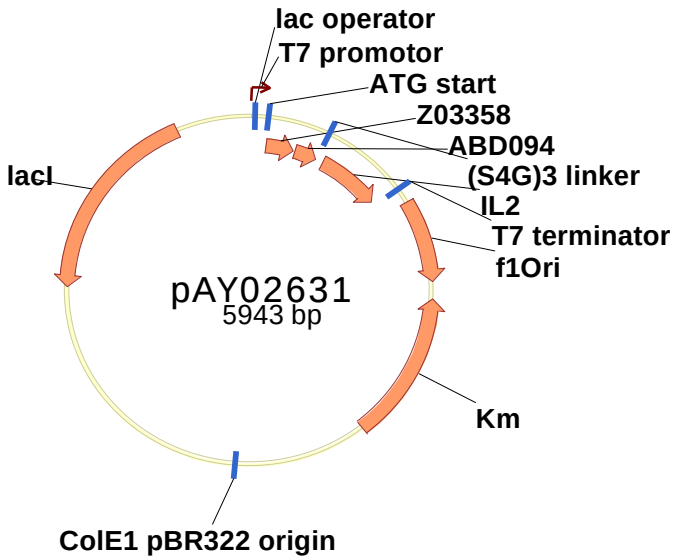
Z03358-ABD094-(S4G)3-IL2 is expected to be insoluble in *E. coli*, both because of its size and previous experience with recombinant production of IL2. Over-expression of the IL2 protein using bacterial expression systems almost always result in inclusion bodies and later purification and refolding is needed. Z03358-ABD094-(S4G)3-IL2 is therefore a good model protein to use when trying to evaluate if it is possible to manipulate an expression system for maximum solubility. His6-(Z05477)2 is an Affibody® molecule that is expressed 50/50 % soluble/insoluble during small scale over-expression using BL21(DE3). His6-(Z05477)2 is also a good model protein to use to determine if it is possible to shift the trend towards more soluble protein production, by testing different expression systems and controlling the cellular milieu.

2 Materials and methods

2.1 Strains and plasmids

Two different plasmid constructs shown in Figure 4 were used during the cultivations, pAY02631 and pAY02610. Two *E. coli* strains have been used for the cultivations, BL21(DE3) (Novagen) and Lemo21(DE3) (Xbrane Bioscience AB). Lemo21(DE3) carries an additional chloramphenicol resistance as well as a gene coding for T7 lysozyme and a promoter rhaBAD[12].

a



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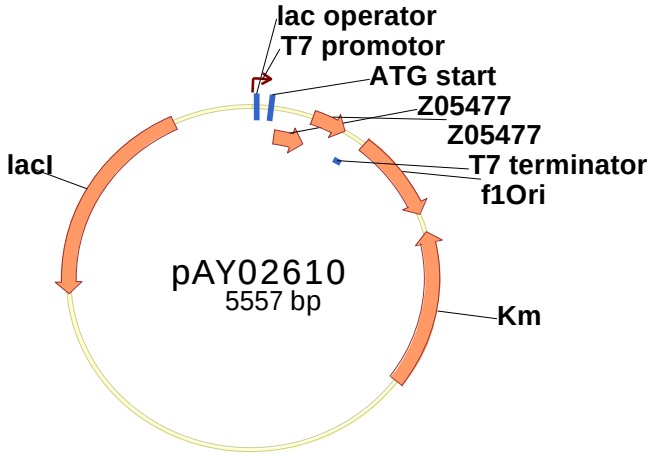


Figure 4. Plasmid map of the constructs pAY02631 (a) and pAY02610 (b). The construct denoted pAY02631 encodes the fusion protein Z03358-ABD094-(S4G)3-IL2 consisting of a PDGFR-β binding Affibody® molecule, fused to ABD and the immunocytokine IL2. The other construct denoted pAY02610 encodes an Affibody molecule that binds a confidential target protein. The constructs carries a kanamycin resistance gene. Both constructs contain a T7 phage promoter, a lactose repressor gene (LacI) and a lactose operator LacO for regulation of expression.

2.2 Transformation

Plasmids were isolated from the *E. coli* host cell by the QIAprep Spin Miniprep protocol (Qiagen) according to the manufacturer's manual and stored in -80˚C freezer.

Competent *E. coli* strains were thawed on ice and 1µL plasmid solution was added and incubated on ice for 1 minute. The constructs were then transformed by electroporation at 1700 V, 200 Ω and 25 µF in 1 mm cyvettes. 1 ml APS-select medium (see Appendix A) was added and the solution incubated at 37˚C for 45 minutes. Transformed cells were spread on APS-select agar plates containing selective antibiotics and incubated at 37˚C overnight.

2.3 Working cell bank (WCB)

One colony per agar-plate (see 2.2 Transformation) was grown in 100 ml APS-select medium with 50 µg/ml kanamycin for both strains and 30 µg/ml chloramphenicol for Lemo21(DE3) (see 2.1 Strain and plasmid). The cultures were grown at 37˚C to an optical density of OD=0.5 at 600 nm (OD_{600}). To minimize adverse effects all cultivations were done in duplicate and all density measurements were done using the duplicates. Standardized tubes were prepared with 300 µl 50 % glycerol and 700 µl culture and stored in the freezer at -80˚C.

2.4 Medium

The medium used in the cultivations were TSB+YE medium (see Appendix A), APS select medium and defined medium (see Appendix A). The defined medium was prepared by first mixing ammonium sulphate, phosphate citrate and de-ionized water. This solution was autoclaved and glucose, trace elements, magnesium sulphate and selective antibiotics were added in a sterile environment. APS-select medium was only used for transformation (see 2.2 Transformation) and when preparing WCB (see 2.3 WCB). TSB+YE medium were used in all cultivations except in fed-batch cultivations, and defined medium was used in all fed-batch cultivations. In the fed-batch cultivations a 50% or 60% glucose feed was started when the added starting glucose was exhausted after 3 h. A predefined feed profile was calculated that reached a plateau after approximately 10h feed.

2.5 Cultivation

Small scale cultivations were performed in both 24 well format with a working volume of 2-10 ml as well as flasks with a working volume of 100-350 ml. Cultivations using a system called SensorDish® Reader (SDR) (PreSens) was conducted to investigate the oxygen consumption. The SDR-system works by measure of oxygen levels in 24-well multidishes. An optical oxygen sensor in the bottom of each well measures the oxygen at a predefined time interval. Large scale fermentations were carried out in two bioreactor systems with working volumes of 6 and 20 liters. All bioreactors were equipped for the control of pH and dissolved oxygen (DO). Both the 6X1 l Greta Multi-fermentor system (Belach) and the 20 l bioreactor (Belach) have an InControl Phantom software enabling control of pH, temperature, feed, aeration, stirring and oxygen supply. Stirrer, temperature and feed profiles were calculated to obtain maximum protein production.

When using the Greta system additional oxygen was connected and used when necessary. Between 0.3-0.9 ml Breox FMT 300 (Cognis) was added to each bioreactor in Greta to reduce foam formation. 5.2 ml Breox FMT 300 was added to the 20 l bioreactor. All optical density measurements were performed with a CO800 Cell Density Meter (Biochrom WPA) at 600 nm (OD_{600}). The samples were diluted with 0.9% NaCl (see Appendix A) to the interval 0.1-1 that lies within the instruments measurable range.

2.6 Protein expression

Several experiments testing different temperatures (27-37˚C), induction OD and expression time were conducted in different previously stated formats (see 2.5 Cultivation). Selection antibiotics were added and depending on strain (see 2.1 Strain and plasmid) kanamycin was added to a concentration of 50µg/ml and chloramphenicol to a concentration of 30 µg/ml. L-rhamnose was added to reach a final concentration of 0-2 mM at the time of inoculation for the small scale cultivations. The cultures were inoculated with thawed 1 % WCB cultures prepared as previously stated (see 2.3 WCB) grown at 200 rpm until the optical density reached OD_{600}=0.4-2 and induced with 400 µM isopropyl β-D-thiogalactoside (IPTG). A Multitron incubator (Infors) and a Termaks incubator (Termaks) were used for incubation in all small-scale cultivations with a 200 rpm stirrer speed.

The fed-batch cultivations were conducted in 20 l and 6x1 l bioreactors and the procedure were as follows. 100 ml defined shake flask medium containing antibiotics was prepared (see Appendix A) and inoculated with 1 % WCB culture. The culture was incubated at 37˚C for approximately 24 hours. The reactors were prepared and inoculated with 1% defined shake flask medium. The temperature was set to 37˚C until start of induction and experiments with expression at 27˚C, 30˚C, 33.5˚C and 37˚C were subsequently conducted. The stirrer profile was set to go from 500-1500 rpm 3 hours after inoculation. The feed profile was set to start 3 h after inoculation, reaching 18, 22.9 and 27.7 g/h after approximately 10h. After 10 hours the culture was induced by automatic induction with 500 µM IPTG. When using Lemo21(DE3) as expression system 0, 225 and 450 µM L-rhamnose was added at the time of induction depending on the experiment. pH was regulated and kept at 7 by ammonia from external flasks. Aeration was kept constant at 1 VVM. When using the 20 l system the culture was induced twice 20.5 and 25.5 hours after inoculation. The cells were harvested 25-30 hours after induction at OD_{600} 50-130 and centrifuged at 15900g for 20 min, 4˚C. The pellets were stored at -20˚C.

2.7 Expression analysis

SDS-PAGE was used for expression analysis and quantification. During all cultivations, samples were taken at regular intervals and OD_{600} was measured. To standardize the procedure for preparing pellets the following procedure was performed. The volume of fermentation broth used was calculated by the formula

$$x = \frac{1000}{OD600}\,[\mu l].$$

From the formula above the volume in µl was calculated with respect to the OD and this means that the same amount of cells in each pellet were prepared and later loaded on the gel. This makes it possible to compare the amount of expressed protein. The sample was

centrifuged at 13 000 rpm, 10 minutes, 4˚C. The supernatant was removed and the pellet containing the sample was stored at -20˚C until expression analysis was performed. The sample was thawed and 150 µl CelLytic™B Cell Lysis Reagent (Sigma) was added and the sample was vortexed for 15 minutes. The sample was centrifuged at 13 000 rpm for 5 minutes, 4˚C. The soluble supernatant and insoluble pellet was separated and 52.5 µl NuPAGE™ Loading Dye Solution (LDS) (Invitrogen) and 22.5 µl 0.5 M DL-1,4-dithiothretole (DTT) was added to both fractions. The samples were heat treated for 20 minutes at 70˚C and then spun down for a few seconds. The samples were loaded on a NuPAGE™ 4-12% Bis-Tris-gel (Invitrogen). 7.5-15 µl sample was loaded on the gel which corresponds to 1/30 to 1/15 of the prepared pellet. 3 µl Novex® Sharp Protein Standard (Invitrogen) was also loaded on the gel. The electrophoresis was run at 200 V, 35 minutes. After electrophoresis the gel was stained with Coomassie staining solution for 1 h, first destained for 1 h with 10 % ethanol 10 % acetic acid, followed by another hour with a 30 % ethanol 10 % acetic acid solution.

2.7.1 Quantification

A standard was prepared by taking uninduced samples from Lemo21(DE3) and BL21(DE3) cultures. The samples were prepared as described before (2.7 Expression analysis) except that only the soluble fraction was used. Different amounts of pure protein were added to the soluble fraction to create standards that had known concentrations of protein. The standards were stored at -20˚C. This procedure was only done for His6-(Z05477)2.

A software program called QuantityOne (Bio Rad) was used to quantify the bands on the gels by densitometry for His6-(Z05477)2. The fusion proteins were quantified by preparing the gel as described in section 2.7 and then estimating the amount of protein by visual inspection.

2.8 Purification

Purification was performed to verify that the correct protein was produced in the cultivations. Only His6-(Z05477)2 was purified and since this was a His-tagged protein, purification with IMAC was used. Pellet was thawed and a small piece was weighed. 25 ml binding buffer (20 mM Sodium Phosphate, 0.5 M NaCl, 20 mM Imidazole, pH=7.4) and 4 µl Benzonase®endonuklease were added. The pellet was vortexed and incubated on ice. The sample was sonicated at 50% amplitude, pulse 5 seconds on 5 seconds off, effective time 1 minute using a Vibracell VC 750 (Sonics) sonicator. The sample was centrifuged at 25 000 g, 8˚C and the supernatant was saved. The sample was then loaded on a pre-prepared His GraviTrap (GE Healthcare) column. After adding 10 ml washing buffer (20 mM Sodium Phosphate, 0.5 M NaCl, 60 mM Imidazole, pH=7.4) the sample was eluted with 3 ml elution buffer (20 mM Sodium Phosphate, 0.5 M NaCl, 500 mM Imidazole, pH=7.4). The sample was quantified using a Nanodrop Spectrophotometer (Saveen Werner).

2.9 Protein analysis

High performance liquid chromatography (HPLC) coupled to mass spectroscopy (MS) was used to verify the size and identity of the purified protein. Samples were prepared as

stated above (see 2.8 Purification). Liquid chromatography with online mass spectroscopy with a narrowbone Zorbax® 300SB-C8 column (2.1*150 mm, 3.5u) was performed and the mobile phase consisted of a gradient of combinations of buffer A and B (CH_3CN/0.1% TFA). The ratio between the two buffers was increased from 10% to 70% of Buffer B during 25 minutes.

2.10 Optimization

A few parameters were chosen as factors to be optimized with the help of MODDE 9.0 (Umetrics) This optimization model was only successfully performed for His6-(Z05477)2 with BL21(DE3) as expression system. The variables varied were temperature, expression time and glucose feed rate. The ranges for the chosen factors are shown in Table 2.

Table 2. Factors chosen for optimization and their ranges.

Factor	**Low**	**Middle**	**High**
Temperature	30˚C	33.5˚C	37˚C
Expression time	6 h	9.5 h	13 h
Glucose feed rate	18 g/h	22.9 g/h	27.7 g/h

3 Results

3.1 Expression of pAY02631 in Lemo2 (DE3) and BL21(DE3)

This section describes the results for expressing Z03358-ABD094-(S4G)3-IL2 with the expression systems Lemo21(DE3) and BL21(DE3).

3.1.1 Characterization of Lemo21(DE3) and determination of the optimal rhamnose concentration

Several small-scale cultivations investigating different rhamnose concentrations and temperatures were conducted to characterize the system. Figure 5 shows that an optimal rhamnose concentration at 500µM and expression at 30°C resulted in the most soluble product and the least insoluble product. However, when continuing the characterization the results were not reproducible and cultivations performed under the same conditions showed mainly insoluble protein. There was more degradation of the product after 22 h compared to after 5h induction. Figure 6 shows an experiment performed under the same conditions but with a different result. In this experiment the result indicated that the protein was insoluble at 30°C regardless of temperature and L-rhamnose concentration.

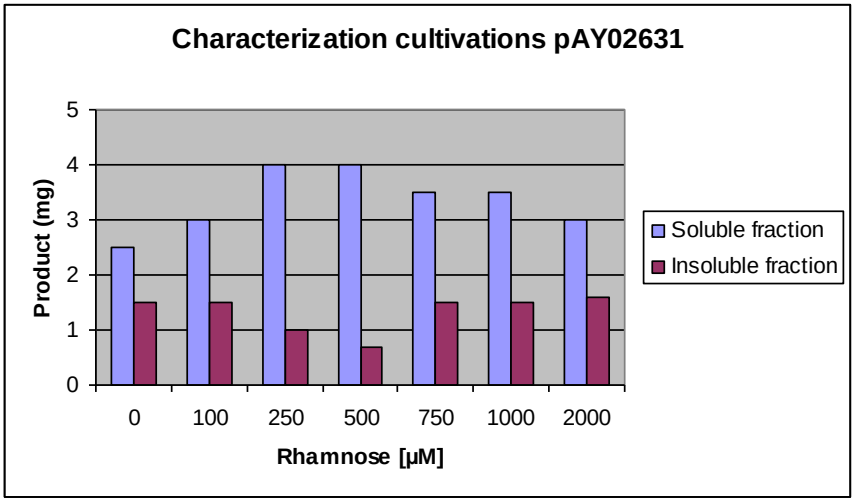


Figure 5. Expression analysis of characterization cultivations 30°C, 5 h induction.

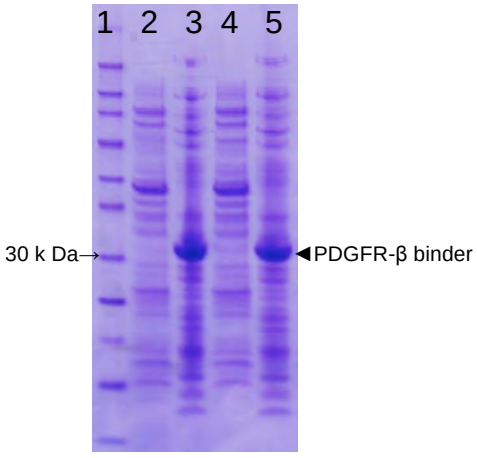


Figure 6. 1;Novex sharp standard (Invitrogen) , 2; [0] µM rhamnose, 3; [0] µM rhamnose, 4; [500]µM rhamnose, 5; [500] µM rhamnose; 30°C, 5.5 h induction, even numbers; Soluble fractions

3.2.1 Fed-batch cultivation in BL21(DE3)

A 13 l fed-batch cultivation of Z03358-ABD094-(S4G)3-IL2 was performed. The protein was produced with a yield of 4.95 g/l but was entirely insoluble as seen in Figure 7. The culture was induced twice and Figure 8, illustrates that maximum OD was reached after 26.5 h, 1 h after the second induction.

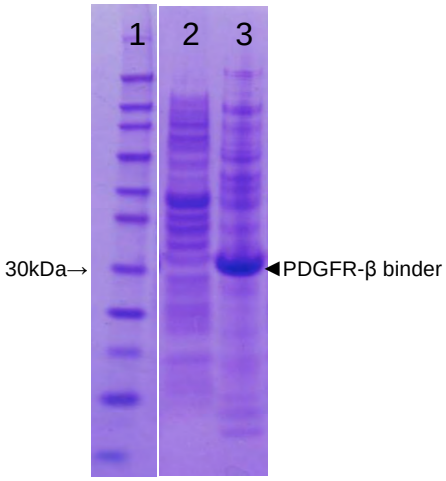


Figure 7 . Expression analysis of the fed-batch cultivation of pAY02631
1; Novex sharp standard (Invitrogen),
2; Soluble fraction,
3; Insoluble fraction, 37°C, 6.5 h induction

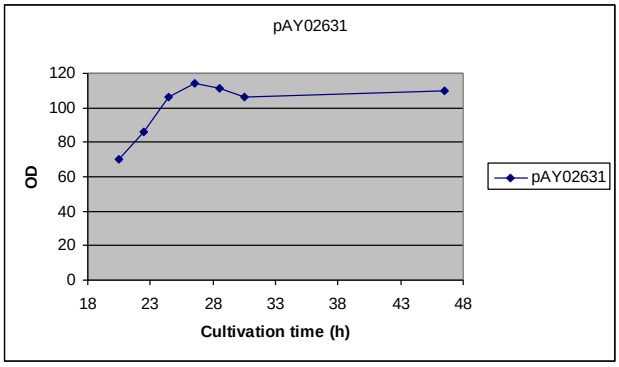


Figure 8. Optical density measurements for the fed-batch cultivation of pAY02610.

3.3 Expression of pAY02610 in Lemo21(DE3) and BL21(DE3)

This section describes the results for expressing His6-(Z05477)2 with the expression systems Lemo21(DE3) as well as BL21(DE3).

3.3.1 Characterization of Lemo21(DE3) and determination of the optimal rhamnose concentration

Several small scale cultivations investigating different rhamnose concentrations and temperatures were conducted to characterize the Lemo21(DE3) system. A comparison to the BL21(DE3) system was made and illustrated in Figure 9. The results in Figure 10, shows that there was less expression of the target protein with increasing rhamnose concentration. The results for longer induction times were not consistent because there was degradation of the product. For a concentration of 250 µM rhamnose there was insoluble product formation and for higher concentrations like 600 µM the product was completely degraded. 30°C was the temperature that consistently gave the most soluble product.

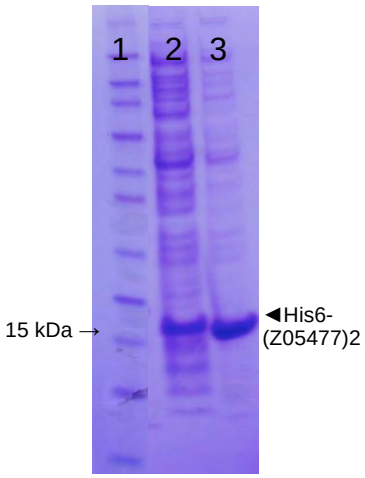


Figure 9. Expression analysis of characterization cultivations; BL21 (DE3) 1; Novex sharp standard (Invitrogen), 2; soluble fraction, 3; Insoluble fraction;30°C, 10 h induction

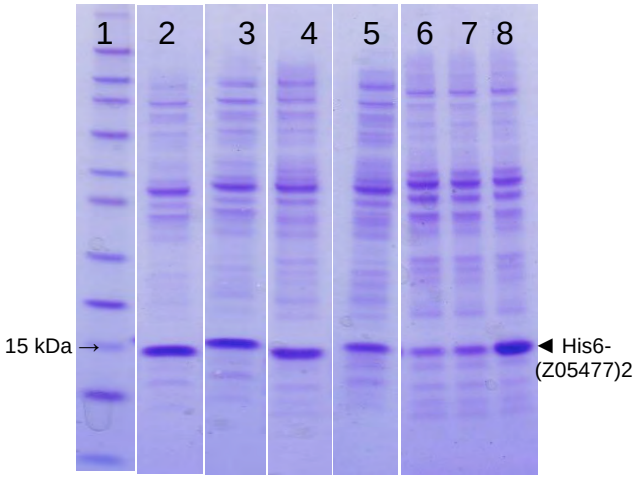
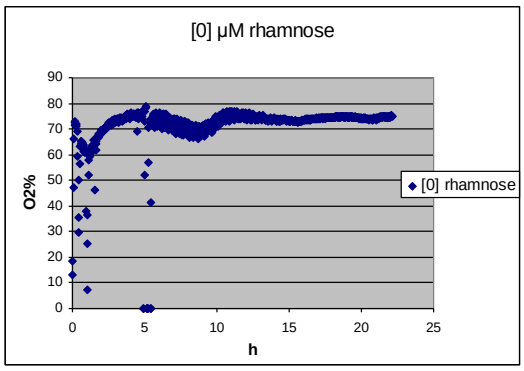
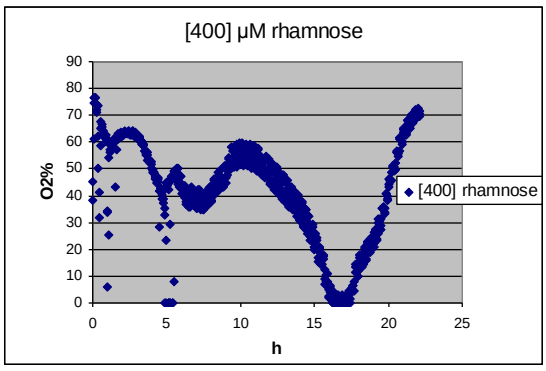


Figure 10. Expression analysis of characterization cultivations; Lemo21(DE3) 1; Novex sharp standard (Invitrogen) 2; 250 µM rhamnose 3; 300 µM rhamnose 4; 450 µM rhamnose 5; 600 µM rhamnose, 6-8; standard; 0.25, 0.5, 2 µg His6-(Z05477)2, soluble fractions, 30°C 6h induction

3.3.2 SDR (PreSens)

Further experiments to characterize the Lemo21(DE3) system was conducted with small scale cultivations. SDR was used to measure the oxygen consumption in 1 ml cultures. Figure 11 shows the oxygen consumption curves obtained from cultivations with the same conditions except with different rhamnose concentrations. The cultures were induced approximately 4.5 h after the measurements started. It was clear that when using 2 mM rhamnose the culture kept growing long after induction indicating that the expression of target protein was not very strong. The culture with 0 µM rhamnose show that the oxygen consumption never increased indicating that expression started at the time of induction. The expression analysis showed that the most soluble protein expression was obtained when using 400 µM rhamnose. The oxygen consumption curve in Figure 11b, shows that the oxygen consumption decreased after induction. Indicating that the culture kept growing but not as fast as when 2 mM was used.





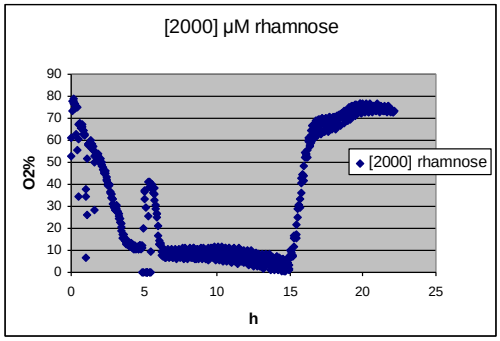


Figure 11a-c. Oxygen consumption curves 30°C, induced after approximately 4.5 h.

3.3.3 Optimization in Lemo21(DE3)

When attempting to optimize the large scale production of His6-(Z05477)2 in Lemo21(DE3) some problems emerged. Only a very small amount of protein was produced during the fed-batch cultivations with Lemo21(DE3). Figure 12 shows that a clear band at approximately 19 kDa could be seen for both 0µM and 450 µM rhamnose but the bands at approximately 15 kDa were very light indicating that there was very low expression levels of the protein. A flask cultivation control was performed with the same inoculums but with a different medium and as lane 4 in Figure 12, shows there was expression of the protein in this culture. BL21(DE3) fed-batch cultivations were run in parallel and the result are shown in Figure 13, illustrating that the protein was soluble.

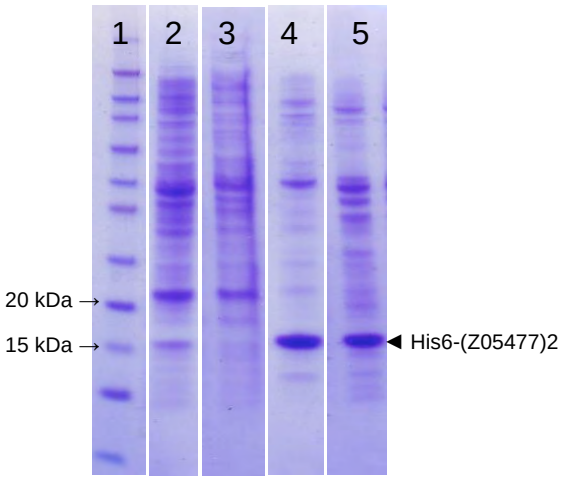


Figure 12. Expression analysis of control and fed-batch cultivation; Lemo21(DE3) 1; Novex sharp standard (Invitrogen), 2; 0 µM rhamnose 3; 450 µM rhamnose; 6h induction 30°C Soluble fractions, 4; control; 3.5 h induction, 37°C, 5; Standard; 2 µg His6-(Z05477)2

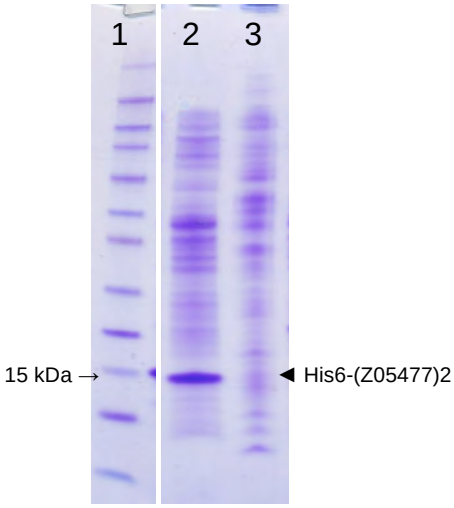


Figure 13. Expression analysis of control; BL2(DE3) 1; Novex sharp standard (Invitrogen), 2; Soluble fraction 3; Insoluble fraction; 6.5 h induction, 30°C

3.4 Optimization in BL21(DE3)

Six fed batch cultivations were done to optimize the production of His6-(Z05477)2 in BL21(DE3) testing different temperatures, glucose feed rates and expression times. Figure 14 shows that most protein was produced in two cultivations with 6 h expression at different temperatures and feed rates. In comparison, there was more soluble product in the cultivation with 18 g/h feed rate and expression at 30°C. The highest yield was 6.6 g/l. The optical density decreased as seen in Figure 15, suggesting some form of degradation of the culture after 9 h induction.

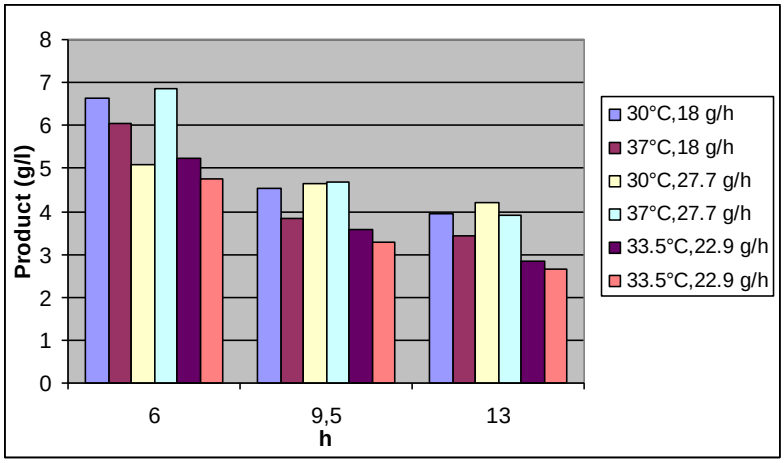


Figure 14. Expression analysis of optimization cultivation with 18 g/h, 22.9 g/h or 27.7 g/h glucose feed.

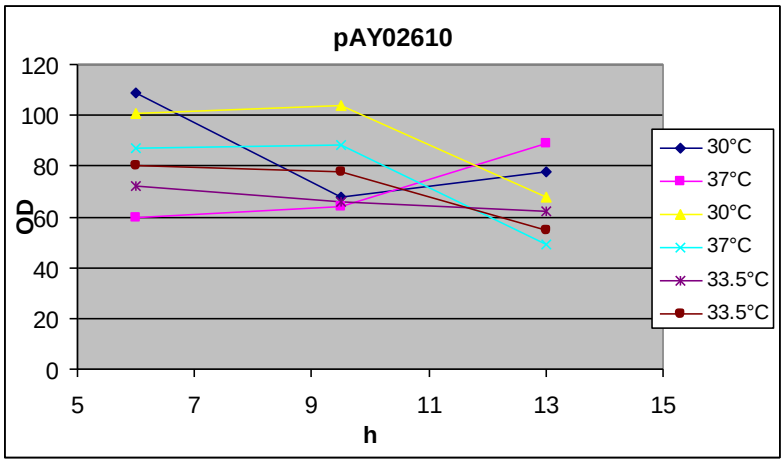


Figure 15. Optical density measurements for the optimization cultivations of pAY02610..

3.4.1 Protein purification

To analyze what was being produced in the fed-batch cultivations with Lemo21(DE3), IMAC purification and HPLC-MS was performed. Nanodrop measurement showed that only 0.2 mg/g pellet was produced. This result proved that the unknown protein at 19 kDa in Figure 8, could not be the target protein migrating differently on the gel but

something entirely different. HPLC-MS show that the purified protein was His6-(Z05477)2 at 14493 kDa (14494 kDa) except glycosylated amino acids at 14671 kDa (178 Da difference indicating glycosylation), as well as minor impurities.

3.4.1 Optimization (MODDE)

R^2 and Q^2 are the two most important terms describing an expression model. The R^2 term shows the model fit and it should be >0.5 for a model with any significance. Q^2 is an estimate of the prediction precision and should be >0.1 for a significant model and >0.5 for a good model. The difference between R^2 and Q^2 should not be more than 0.3. Removing non-significant model terms and choosing the correct transformation results in a better model, and Q^2 is the most sensitive indicator. We tested this on our expression system. The model term feed rate and subsequent terms temperature*feed rate and expression time*feed rate were found to be insignificant model terms. After exponential transformation for solubility and logarithmic transformation for product formation, R^2 was 0.87 for solubility and 0.68 for product formation. Q^2 was 0.75 for solubility and 0.43 for product formation. Summaries of the model statistics are shown in Figure 16. The optimizer gave an estimated expression of approximately 5.3 g/l with almost maximum solubility at 30°C and 6 h expression time. In Figure 17, the optimized contour plots are shown. Data from the optimization cultivations evaluated in MODDE (Umetrics) can be found in appendix B.

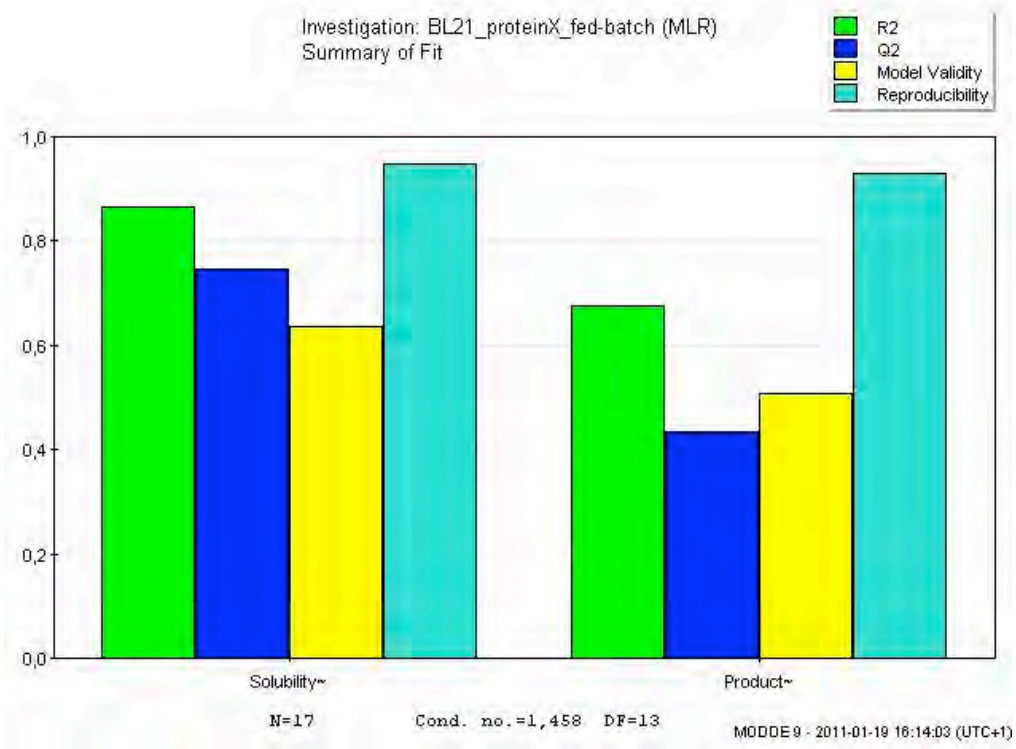


Figure 16. Summary plot of the model statistics.

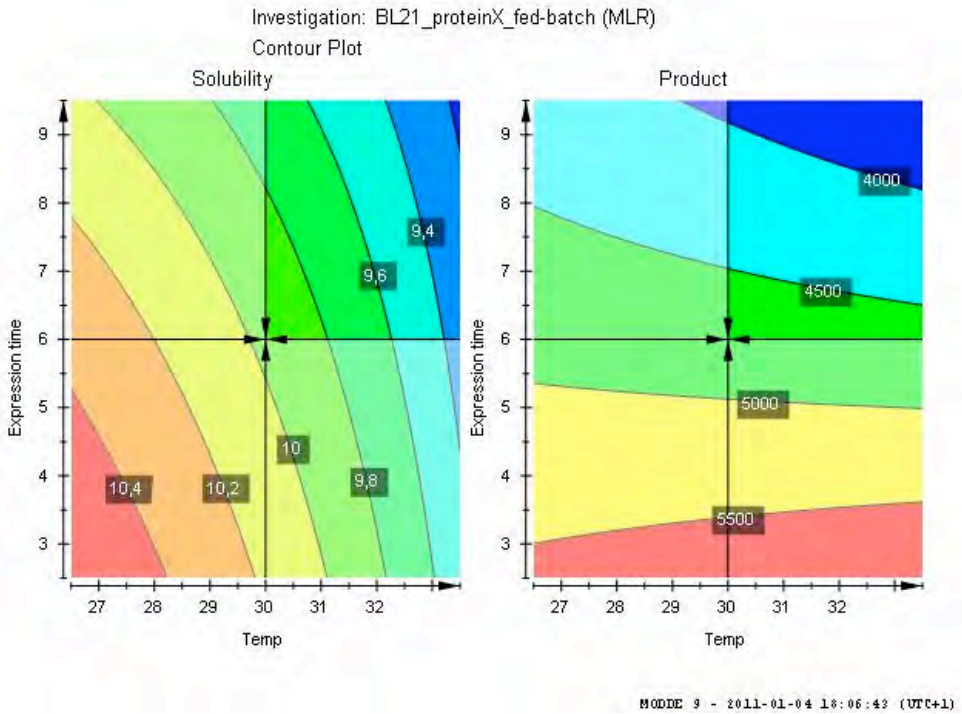


Figure 17. Response contour plot from the optimization cultivations of pAY02610 in BL21(DE3).

4 Discussion

The aim of this project was to optimize the procedure for soluble production of the fusion protein Z03358-ABD094-(S4G)3-IL2 with Lemo21(DE3) *E. coli*. It is hard to purify this protein from inclusion bodies and regain the biological activity. Efforts were therefore made to find an expression system that allowed soluble expression of the protein. Lemo21(DE3) was used because of its tunable promoter system. The main part of the project was characterization of the Lemo21(DE3) system in small scale. There were however problems with the reproducibility. Therefore another protein His6-(Z05477)2 was chosen for expression in Lemo21(DE3). Earlier experiments had shown that this protein is expressed as 50% soluble and 50% insoluble protein in BL21(DE3) in small scale.

When producing IL2 with recombinant protein production it is almost always insoluble but at the beginning of this project the results indicated that Lemo21(DE3) could be a possible expression system for soluble expression of the fusion protein. Early results showed that a concentration of 500 µM rhamnose at 30°C gave soluble expression of the protein. After repeated attempts it was clear that the same conditions sometimes resulted in insoluble expression. Instead the BL21(DE3) system was used for large scale production of the fusion protein with good results in respect to amount of protein produced but completely insoluble with a yield of 4.95 g/l at 37°C.

The quantification method used in the beginning of the project was to measure the intensity of SDS-PAGE bands by eye measure. A better quantification method could have been used like densitometry but this does not have much effect on the result in this case. The aim was to produce the protein in soluble form and even without a better quantification method it was easy to see the relative comparison between the soluble and insoluble protein on the gels. The inoculums varied between the cultures when working with Z03358-ABD094-(S4G)3-IL2 since the WCB cultures had not yet been prepared and this might have had an effect on the reproducibility of the cultivations.

Since the expression of soluble protein was not successful another protein was investigated. The project proceeded with the production of the protein His6-(Z05477)2, which had been expressed 50/50 % soluble/insoluble in BL21(DE3). The results comparison between Lemo21(DE3) and BL21(DE3) showed that it was possible to use Lemo21(DE3) for small scale production to eliminate the insoluble fraction. It was clearly not possible to use Lemo21(DE3) for production of His6-(Z05477)2 with HCDC. BL21(DE3) gave excellent results in HCDC with a yield of 6.6 g/l soluble protein.

There were inconsistencies with questions concerning degradation and variances between production levels at the same rhamnose concentrations. This could have been better dealt with if replicas of the experiments had been used. The determination of the optimal rhamnose concentration was complicated by the degradation of the product after longer expression times. It was therefore easier to determine optimal rhamnose concentration for shorter induction times. A rhamnose concentration at 450 µM and expression at 30°C seemed the best choice for Lemo21(DE3) in small scale. The quantification method with

densitometry should be sufficient for approximate quantification, at least in determining the soluble fraction relative the insoluble fraction.

The results from the SDR measurements are perhaps not reliable for longer expression times since the cultivation volumes of 1 ml mean that the growth was limited. To determine if the problems with expression in Lemo21(DE3) in fed-batch cultivations were due to elimination of the plasmid containing the protein sequence, controls with the same inoculums were made and showed that this was not the case. As the results show in section 3.3.3 there was some expression with 0 μM rhamnose concentration but none for 450μM. It is possible that the repressor T7Lys is constantly expressed in the fed-batch cultivations leading to low expression levels. The adding of rhamnose at the time of induction may have completely inhibited expression of the protein of interest. Since there was very low expression of the protein, also with 0μM rahmnose concentration, the results indicate that the problem lies within the Lemo21(DE3) system.

Another possibility could be to use the Rhamex system (Xbrane Bioscience AB), that shows similar properties as Lemo21(DE3). This system works in a more direct fashion. When adding rhamnose the rhaBAD promoter is activated. The rhaBAD DNA sequence have been replaced with a cloning site that allows any gene to be placed under the control of the rhamnose inducible promoter[13]. It is possible that the problems with fed-batch cultivations could be eliminated with this tighter control of expression. If the problems met with fed-batch in Lemo21(DE3) were due to constantly activated rhaBAD promoter and accumulation of T7Lys, then the Rhamex system could be an interesting alternative for optimization.

The small scale cultivations of His6-(Z05477)2 in BL21(DE3) showed that it was less than 50 % soluble compared to insoluble. Surprisingly in fed-batch the protein proved soluble. The results from the fed-batch cultivation in BL21(DE3) showed that His6-(Z05477)2 was expressed in high levels with 6.6 g/l yield soluble protein. Again the relative amounts of protein produced in soluble and insoluble fractions were more easily determined than the actual quantity of produced protein. It is safe to say that the amount of soluble His6-(Z05477)2 produced in the fed-batch cultivations was quite high.

The optimization in MODDE (Umetrics) was actually more of a screening process because of lack of time. The software was still able to produce an optimum at 6 h expression at 30°C. The feed rate was deemed non-significant but this could be due to problems with the model because of degradation of the product. This would need to be further investigated to draw that conclusion. If the temperature is excluded from the factors for product formation the optimum ends up at a higher temperature but the same expression time. This is probably due to the compromise between what is the optimum for the different responses. To optimize the procedure further a set of optimization cultivations should be performed, where the lower range of expression times and temperature is tested. The red area in the lower part of Figure 13, shows that the true optimum could lie in that region.

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Appendix A

APS-select medium:
20 g/l BBL™ Select APS™ LB Broth Base (BD Becton Dickinson).

Tryptic soy broth and Yeast extract medium (TSB+YE):
30 g/l Tryptic Soy Broth (Merck) and 5 g/l Yeast extract (Merck).

Defined medium:
3.75 g/l $(NH_4)_2SO_4$, 0.72 g/l $MgSO_4$, 3 g/l K_2HPO_4, 4.5 g/l KH_2PO_4, 1.85 g/l $Na_3C_6H_5O_7 \cdot 2H_2O$, 0.053 g/l $FeCl_3 \cdot 6H_2O$, 0.016 g/l $ZnSO_4 \cdot 7H_2O$, 0.004 g/l $CuSO_4 \cdot 5H_2O$,0.2 g/l $MnSO_4 \cdot H_2O$ and 0.021 g/l $CaCl_2 \cdot 2H_2O$, 1 g/l 50/60 % glucose.

Defined shake flask medium:
6.7 g/l YNB (Difco™ Yeast Nitrogen Base without amino acids, Becton Dickinson), 5.5 g/l glucose, 7 g/l K_2HPO_4, 1 g/l $Na_3C_6H_5O_7 \cdot 2H_2O$.

Appendix B

Table 3. Data used for optimization in MODDE

Fermentor	Strain	Temp °C	Feed after induction g/h
H1	BL21	30	18.05
H2	BL21	37	18.05
H3	BL21	30	27.756
H4	BL21	37	27.756
H5	BL21	33.5	22.903
H6	BL21	33.5	22.903

Table 4. Cultivation data used for optimization in MODDE

Fermentor		Volume (ml)	Pellet (g)	OD 6 h	Product (mg)	Product (g/l)
H1	pAY02610	878	67,1	109	5828	6,6
H2	pAY02610	846	105,2	60	5117	6,0
H3	pAY02610	903	116,5	101	4597	5,1
H4	pAY02610	740	58,3	87	5060	6,8
H5	pAY02610	884	74,6	72	4621	5,2
H6	pAY02610	858	72,3	80	4077	4,8
	Construct	**Volume (ml)**	**Pellet (g)**	**OD 9.5h**	**Product (mg)**	**Product (g/l)**
H1	pAY02610	878	67,1	68	3994	4,5
H2	pAY02610	846	105,2	64	3232	3,8
H3	pAY02610	903	116,5	104	4198	4,6
H4	pAY02610	740	58,3	88	3477	4,7
H5	pAY02610	884	74,6	66	3151	3,6
H6	pAY02610	858	72,3	78	2811	3,3
	Construct	**Volume (ml)**	**Pellet (g)**	**OD 13h**	**Product (mg)**	**Product (g/l)**
H1	pAY02610	878	67,1	78	3452	3,9
H2	pAY02610	846	105,2	89	2914	3,4
H3	pAY02610	903	116,5	68	3776	4,2
H4	pAY02610	740	58,3	49	2883	3,9
H5	pAY02610	884	74,6	62	2499	2,8
H6	pAY02610	858	72,3	55	2265	2,6