



Bachelor's thesis

Protein production and purification of type II transmembrane protease Tmprss2 in *Pichia pastoris* (*Komagataella phaffii*)

Datum: 25.08.2017

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Summary

The purpose of this Bachelor's thesis "Protein production and purification of type II transmembrane protease Tmprss2 in *Pichia pastoris* (*Komagataella phaffii*)" is to assess optimization possibilities for production and purification methods of mouse Tmprss2 in *Pichia pastoris*. The aim of the thesis was to develop upstreaming and downstreaming strategies capable of producing 2 mg amounts of properly folded and active Tmprss2 for crystallization experiments. Solving the crystal structure of Tmprss2 and the enzyme-substrate interaction surface in combination with Hemagglutinin 1 could lead to determining the process of proteolytic activation of Hemagglutinin 1 by Tmprss2. Therefore producing Tmprss2 for crystallization is an essential step for structure-based drug design for Hemagglutinin 1. This thesis is specifically concerned with the cultivation of recombinant *Pichia pastoris* cell lines in 2 L scale using a bioreactor to demonstrate the potential of iterative methanol induction cultivation mode for Tmprss2 production. The initial hypothesis was that the production of Tmprss2 could be increased with optimized cultivation methods and NiSephacel™ Excel resin material could be utilized in optimizing direct immobilized metal ion affinity chromatography (IMAC) of Tmprss2. It is important to note that for IMAC fusing Tmprss2 with a histidine-tag is required, consequently recombinant expression is necessary for performing IMAC.

The iterative methanol induction cultivation mode has been previously used in Tmprss2 production in flask scale successfully. Based on previous studies advanced strategies for upscaling the iterative methanol induction cultivation mode to reactor scale were developed and tested to meet production goals for crystallization. The scope of the experiments was limited to developing optimized cultivation strategies for previously developed recombinant *Pichia pastoris* cell lines and exploring the potential of NiSephacel™ Excel resin material in immobilized metal ion affinity chromatography of Tmprss2. The previously developed cell lines used in this thesis work were "Tmprss2-Mut" (*P. pastoris* KM71H/pPICZ α -Tmprss2-D343Nopt clone o108 MutS) and "Tmprss2-WT" (*P. pastoris* Wild type jackpot clone KM71H-pPICZ α -Tmprss2-WTopt o20). The research was carried out in five experimentation Cycles over the period of 11 weeks in "Recombinant Protein Expression"-research group in Helmholtz-Centre for Infection Research, Braunschweig, Germany.

The outcome of this research into protein production and purification of type II transmembrane protease Tmprss2 in *Pichia pastoris* was a strategy for optimized cultivation of *Pichia pastoris* cell lines in 2 L scale using a bioreactor in iterative methanol induction cultivation mode. The initial hypothesis was also confirmed as NiSephacel™ Excel resin material could be utilized in direct immobilized metal ion affinity chromatography of Tmprss2 without issues with

column stripping common in similar IMAC materials designed for histidine tagged proteins. Tmprss2 was produced in the target amount for crystallization experiments in a single iteration using the Tmprss2-Mut cell line. However, the fast 48 h production Cycle in iterative methanol induction mode would allow pooling the produced Tmprss2 from several successive iterations to reach higher amounts. Pooling the products of several iterations would make reaching sufficient amounts for crystallization of the desired autocatalyzed form of Tmprss2 produced by the Tmprss2-WT possible. The issue with the lowered Hemagglutinin 1 cleaving activity of Tmprss2 D343N produced by the Tmprss2-Mut expression cassette could potentially be countered by coincubation with processed Tmprss2 produced with the WT-Tmprss2 expression cassette. Autocatalyzing the uncleaved Tmprss2 D343N to process it to the truncated form with higher activity would reduce the amount of iterations required to reach ample amounts of active Tmprss2 for crystallization.

The limitations of the developed upstreaming and downstreaming strategies were also recognized. The main issues encountered were loss of Tmprss2 in concentration and through degradation over time. The purification using IMAC was prone to issues with precipitation of the culture supernatant interfering with the process. The iterative methanol induction cultivation mode requires removing the cell culture from the reactor in between iterations causing increased risk of contamination. Further development for countering these limitations were suggested in the form of modified techniques. Loss of protein during concentration might be avoided using a more sophisticated concentration technique i.e. repeated IMAC, diafiltration or TCA precipitation. The degradation of Tmprss2 could be limited by conducting IMAC in 4 °C instead of room temperature. The precipitation problems may be addressed by monitoring and adjusting the concentration of media components throughout the cultivation to prevent building up of materials in successive iterations. Risk of contamination and degradation of Tmprss2 during cultivation could be decreased by implementing continuous cultivation with perfusion membrane for harvesting. The cultivation strategy could also be shifted from aiming to shorten the glycerol growth phase to aiming to run the reactor in ultra-high cell density conditions. Running the reactor in the range of OD > 500; WCW > 400 g/L would be a trade-off for increased Tmprss2 total yields at the cost of longer process time for a single batch of Tmprss2. The increased total yields could be reached by upscaling the reactor cultivation based on the methods developed for a 2 L scale bioreactor in iterative methanol induction cultivation mode.

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Abbreviations and terms

AF	Anti-foam, 5% (V/V) Struktol J 673 dissolved in methanol
AOX1	Alcohol oxidase 1 promoter
BMGY	Buffered minimal glycerol medium with yeast extract
BMMY	Buffered minimal methanol medium with yeast extract
GRAS	Generally recognized as safe
Cycle 0	Tmprss2-WT in 2 L Erlenmeyer flasks in fed-batch cultivation mode
Cycle 1	Tmprss2-WT in 2 L Erlenmeyer flasks in hybrid iterative methanol
Cycle 2	Tmprss2-WT in 2 L Labfors 3 reactor in iterative methanol induction cultivation mode
Cycle 3	Tmprss2-Mut in 2 L Labfors 3 reactor in iterative methanol induction cultivation mode
Cycle 4	Tmprss2-WT in 2 L Erlenmeyer flasks in single batch cultivation mode
GOI	Gene of interest
H1	Hemagglutinin 1
HAT / DESC	human airway trypsin-like protease/differentially expressed in squamous cell carcinoma subfamily
HZI	Helmholtz-Centre for Infection Research, Braunschweig, Germany
HPI	Hours post induction
IMAC	Immobilized metal ion affinity chromatography
<i>in vitro</i>	In a controlled environment outside of a living organism in a reaction tube or bottle
<i>in vivo</i>	In whole living organisms or cells
kDa	Kilodalton
Milli-Q water	Ultrapure water of Type 1 as defined in ISO 3696:1987

MWCO	Molecular weight cut-off
OD ₅₉₅	Optical density at 595 nm wavelength
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pO ₂	Dissolved oxygen
<i>P. pastoris</i>	<i>Pichia pastoris</i> (<i>Komagataella phaffii</i>)
rpm	Rounds per minute
RT	Room temperature 23 °C on average
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
S1	Laboratory biosafety level one
TEV protease	Tobacco Etch Virus nuclear-inclusion-a endopeptidase
Tmprss2	Transmembrane protease, serine 2
Tmprss2 D343N	a form of Tmprss2 produced by Tmprss2-Mut. Mostly in the form of the full ectodomain in 45 kDa range
Tmprss2-Mut	<i>P. pastoris</i> KM71H/pPICZα-Tmprss2-D343Nopt clone o108 mutS-strain
Tmprss2-WT	<i>P. pastoris</i> Wild type jackpot clone KM71H-pPICZα-Tmprss2-WTopt o20
TTSP	Type II transmembrane serine proteases
UV	Ultraviolet
WCW	Wet cell weight in grams of cells per 1 kg of culture (g/kg)
WB	Western blot
YPD	Yeast extract peptone dextrose

1 Introduction

Influenza(H1) is an acute viral infection causing seasonal epidemics especially during wintertime. It is also the cause for several pandemics such as the Spanish Flu A H1N1 (1918-1919), Asian Flu A H2N2 (1956-1958), Hong Kong Flu A H3N2 (1968-1969) and A H1N1/09 Flu Pandemic (2009–2010).

Influenza is mostly non-life-threatening however, it is a general burden to public health and may cause complications leading to death especially in children, the elderly and people with already weakened immune systems. Influenza virus is classified into three genera A, B and C. Originally an avian virus the influenza virus A as can be seen from the list of pandemics is commonly the genus causing pandemics while mammalian viruses B and C cause epidemics on a yearly basis. Bacterial antibiotics are not effective in treating influenza as it is a viral infection (1,2,3).

Instead treatment approaches target neuraminidase and M2 viral proteins involved in replication of the influenza virus. The high mutation rate of H1 is observed to develop resistance to anti-viral drugs hindering their effectiveness. Anti-viral drugs targeting neuraminidase are currently not effective in treating influenza (1,2,4).

Influenza remains a global health concern because of the annual influenza epidemics, the risk of a pandemic due to a new potent mutant and the lack of efficient treatment.

Several reports speculate the transmembrane protease, serine 2 (Tmprss2) to be involved in the activation and subsequent replication of H1 (5,6,7,8). The link has been further studied and Tmprss2 has been found to be vital in the activation of H1 in living mice (9). The link between Tmprss2 and H1 infection was demonstrated again in Tmprss2 deficient mice, which were found to be less susceptible to get infected than WT mice (10, 19). Structural and functional analyses of crystallized Tmprss2 would be required in understanding both its physiological and H1 activation functions. The crystal structure of Tmprss2 and the enzyme-substrate interaction surface in combination with H1 could lead to determining the process of proteolytic activation of H1 by Tmprss2. This would require successful crystallization of active Tmprss2 with H1.

Producing crystals of pure Tmprss2 would require minimum amount of approximately 2 mg of properly folded and active Tmprss2. Tmprss2 is a difficult to express protease because it's produced in extremely low amounts and only

in optimized cell lines of *P. pastoris* among several tested expression hosts. Comparably higher amounts are only reached in mutant D343N form with most of the produced tmprss2 secreted as its full ectodomain at a state with reduced activity with H1 (12). Difficult to express proteases expressed in a heterologous expression system may not be fully soluble, well-folded or in an active form especially when proteases of higher eukaryotes are produced by prokaryotes. Therefore, an expression system capable of performing post-translational modifications resulting in functionally active forms because of mannose type N-glycosylation close to higher eukaryotes and efficient protein folding is required.

Komagataella phaffii previously assigned as *Pichia pastoris* is well known as one of the prominent expression systems in biotechnology (11). This methylotrophic yeast meets the mentioned requirements and is a fast growing and cost effective alternative to mammalian or insect cell cultures. Recombinase mediated cassette exchange *Pichia pastoris* master cell lines for producing the active WT form and mutant form D343N of Tmprss2 were also recently developed (12).

This bachelor's thesis work is focused on creating strategies for producing the difficult to express transmembrane serine protease mouse Tmprss2 in *Pichia pastoris* in sufficient amounts for crystallization experiments.

Upstreaming as well as downstreaming strategies were developed for increasing the mutant (D344N)- and active Tmprss2 yields. Upstreaming strategies were optimized by cultivation of both *Pichia pastoris* cell lines in 2 L scale using a bioreactor in iterative methanol induction cultivation mode. Downstreaming was improved by testing the viability of NiSepharose™ Excel material for direct immobilized metal ion affinity chromatography of Tmprss2.

1.1 The type II transmembrane serine protease Tmprss2

Tmprss2 belongs in the family of type II transmembrane serine proteases (TTSP), which are expressed initially as single-chain proenzymes. TTSP family proteases require autocatalytic activation by the cleavage of their respective conserved zymogenic site (13).

This family has four subfamilies the Matriptase subfamily, the HAT / DESC human airway trypsin-like protease/differentially expressed in squamous cell carcinoma subfamily, the Hepsin / Tmprss subfamily and finally the Corin subfamily. Tmprss2 belongs to the Hepsin / Tmprss subfamily which is differentiated from the other subfamilies by its serine protease domain and its domain structure (14, 15, 16, 17). Regardless of similarity to other proteases in its family Tmprss2 is the only one capable of cleaving H1HA *in vivo* (9). Tmprss2 can be found in a variety of truncated forms, most notably as the active serine protease domain by itself or as the full ectodomain (18).

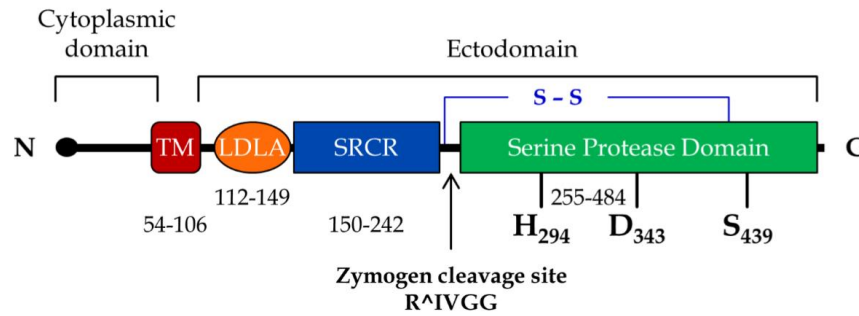


Figure 1 Scheme of Tmprss2 construct; transmembrane domain (TM); low-density lipoprotein receptor class A domain (LDLA; a scavenger receptor cysteine-rich domain (SRCR); C-terminal serine protease domain (SPD). Image from (12)

Tmprss2 as a transmembrane protease is composed of cytoplasmic, transmembrane and ectodomain. The cytoplasmic domain is composed of N-terminal tail which is connected to the ectodomain by its transmembrane domain (TM). The extracellular domain comprises a low-density lipoprotein receptor class A domain (LDLA), a scavenger receptor cysteine-rich domain (SRCR) and the C-terminal serine protease domain (SPD). On Western blot the primary antibody used targets only the C-terminal fragment. Differentiation between the cleaved and unprocessed forms of Tmprss2 was still possible by the molecular weight of the two forms. The total ectodomain prior to autocatalytic cleavage at marked zymogen cleavage site in Figure 1 has a molecular weight of ~45 kDa. The C-terminal serine protease domain (SPD) itself post cleavage has a molecular weight of ~30 kDa. (12)

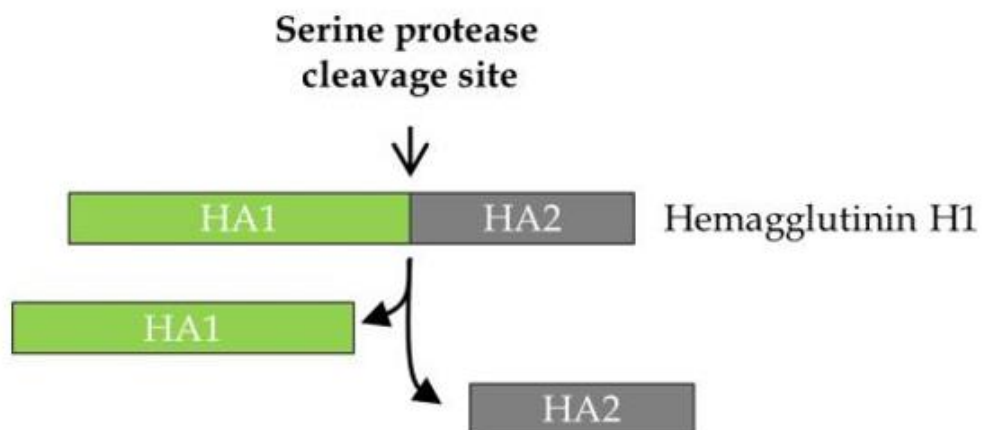


Figure 2. Serine protease cleavage of H1; hemagglutinin H1 from its unprocessed form into two subdomains HA1 and HA2. Image from (12)

Tmprss2 has been speculated and observed to be involved in replication and activation of H1 as previously mentioned in 1 Introduction (5, 6, 7, 8, 9, 10, 19).

Tmprss2-WT and the activity knockdown mutant Tmprss2-D343N have been shown *in vitro* to be able to perform cleavage of H1 as seen in Figure 2. The recombinant D343N expressed mostly as the total extracellular domain of Tmprss2 performs H1 cleavage at a slower rate and it has not been established if it needs to be autocatalytically processed before binding to H1. (12)

Despite demonstrated activity with H1 the physiological function of Tmprss2 remains unclear. The Tmprss2 deficient mice were healthy, showed no changes in their phenotype and had no change in the regulation of their other TTSPs to compensate the lack of Tmprss2 (20).

1.2 Methylophilic yeast *Pichia pastoris*

Komagataella phaffii is a single-cell eukaryotic expression system frequently used to produce secreted eukaryotic proteins. *Komagataella phaffii* assigned as *Pichia pastoris* previously, was relatively recently reassigned as its own separate species. Multigene sequence analysis was used to differentiate *K. pastoris*, *K. phaffii* and *K. pseudopastoris* as separate species of *P. pastoris*. *K. phaffii* was identified as the *Pichia pastoris* strain which is commonly used in gene expression utilizing the “Pichiapastoris Expression Kit” from Invitrogen Corporation (11). Because *Pichia pastoris* is still such a prominent name used in the field of biotechnology to refer to *K. phaffii* in this bachelor’s thesis work *K. phaffii* is also simply referred to as *P. pastoris*.

Unlike prokaryotes, *P. pastoris* can perform post-translational modifications resulting in functionally active forms because of mannose type N-glycosylation close to higher eukaryotes and efficient protein folding. These attributes make it proficient in producing hard to express recombinant proteases such as Tmprss2 (21).

P. pastoris secretes only a few proteins into the culture medium. This leads to a situation where the excreted recombinant protease is the only protease in significant amounts in the supernatant (22). It also has no tendency for hyperglycosylating proteins during posttranslational modification which leads to fewer problems with secretion and/or inactive forms. (23, 24, 12).

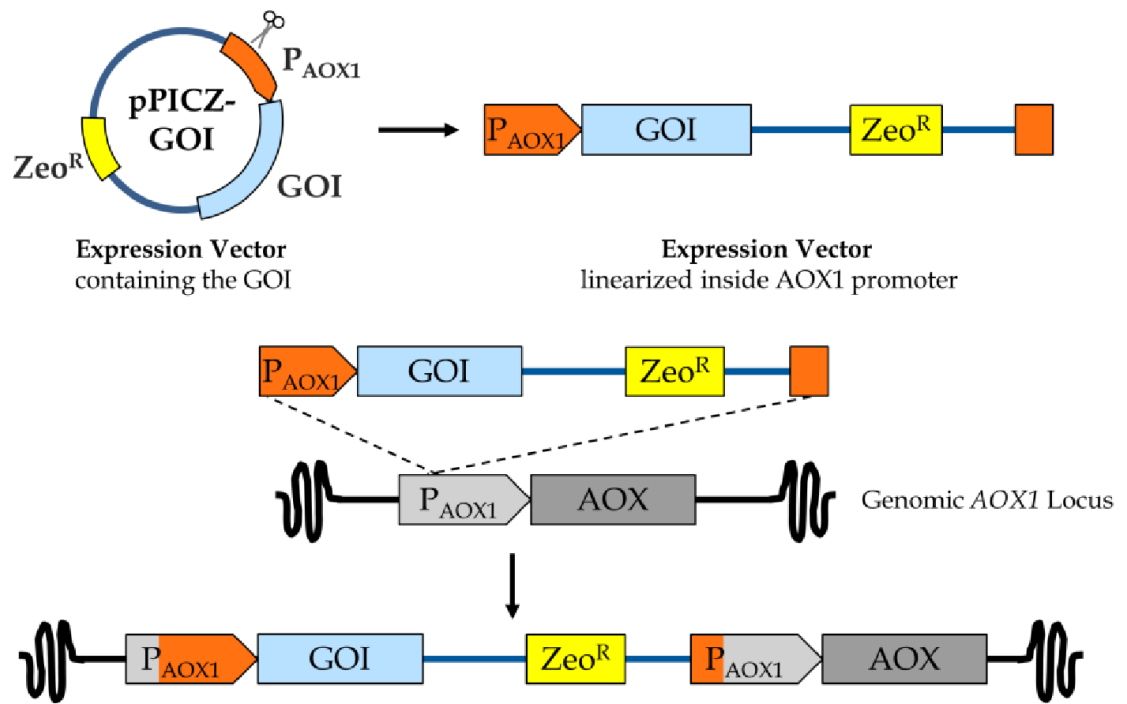


Figure 3. Stable transformation of *P. pastoris* by homologous recombination. Image from (12). Expression cassette for the gene of interest (GOI) with Zeocin resistance is engineered for a recombinant producer cell line. The GOI, the AOX1 promoter “P_{AOX1}” and a Zeocin resistance Zeo^R are in contained expression vector pPICZ-GOI. A uniquely cutting restriction enzyme marked as a pair of scissors on top left linearizes the vector inside “P_{AOX1}”. The vector is inserted into the AOX1 genomic locus homologous to the targeting sequence on the vector by homologous recombination in the yeast nucleus (12).

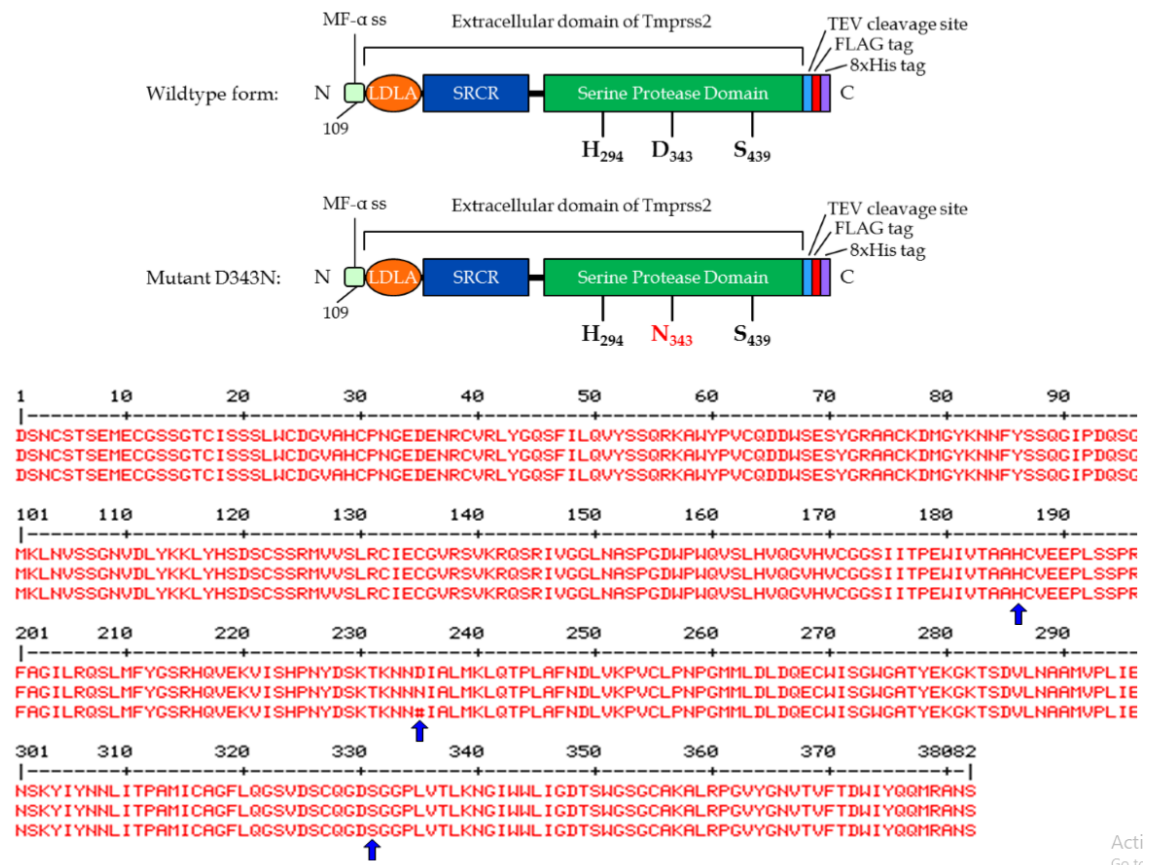


Figure 4. Tmprss2 constructs schematics for recombinant expression in *P. pastoris* cell lines WT and Mut D343N. Image from (12) The protein is directed to the supernatant by N-terminally fusing it with a mating factor α secretion signal (MF- α ss). For purification and detection, a Flag tag and an 8xHis tag are C-terminally fused to the protein. TEV protease site is located upstream of the tags. The function of TEV protease section is to cleave the tags off. Low-density lipoprotein receptor class A domain (LDLA) and a scavenger receptor cysteine-rich domain (SRCR) remain unaltered. Position 109 of the full protein: alignment of the translated recombinant construct sequences used in Tmprss2. Position 235 of the full protein: D343N mutation amino acid replacement of the aspartic acid of the catalytic triad to asparagine. The catalytic triad amino acids are marked with blue arrows (12).

The mutant strain of *P. pastoris* Tmprss2-Mut (Mut D343N in Figure 4.) used in this bachelor's thesis work represents the Methanol Utilization Slow (Mut^s) phenotype. Mut^s strains are characterized by the deletion or disruption of their AOX1 gene as it is replaced by the expression cassette. Their growth on methanol as carbon source is slowed down as the Mut^s strains can only rely on the weaker AOX2 in alcohol oxidase for methanol utilization. The slower growth rate sets a requirement for tightly controlled methanol feed rate maintaining 0,2-0,8% (V/V) methanol in reactor conditions. In comparison to Methanol Utilization Positive (Mut⁺) strains where both AOX1 and -2 promoters are intact this lower feed rate results in slower growth and target protein production, but also lowers the risk of accumulating formaldehyde ultimately resulting in loss of culture. In comparison Mut⁺ strains, Mut^s strains boast a slower, but less risky alternative capable of utilizing the strongly inducible AOX1 promoter for controlling expression. The third alternative Methanol Utilizing Negative Mut⁻ strain

cannot utilize methanol for alcohol oxidase at all because they have both AOX1 and -2 disruption. They are still capable of having their AOX1 promoter induced by methanol in low concentrations. Expression in Mut⁻ strains is conducted with a mixed feed where glycerol is fed at a low rate which does not repress the AOX1 promoter being induced by a constant concentration of methanol which is only being fed to counter the amount lost through evaporation (28).

P. pastoris was cultivated with several different methods throughout this bachelor's thesis work. Regardless of the differences between methods some aspects of the cultivation process remain the same for all methods. The experiments for this thesis work could be carried out in S1 Laboratories as *P. pastoris* is classified to be a GRAS organism. In addition to being safe to work with the cultivation conditions can be described as mild and media components required are relatively cheap. Two main challenges in the cultivation process were the repeated handling of highly inflammable and cytotoxic methanol in quantities up to several litres as well as ensuring the sterility of *P. pastoris* cultures. As a single cell eukaryote, *P. pastoris* is a fast-growing organism and can be cultivated in high cell densities up to 500 OD₅₉₅ in reactor conditions.

This methylotroph is commonly grown in defined simple medium while being fed with carbon sources in phases. This stepwise cultivation strategy is made viable by the yeast carbon source hierarchy of preferably utilizing in descending order first glycerol, then ethanol, acetate and finally methanol. The hierarchical utilization of carbon sources enables very specific induction just by altering the carbon source feed.

The phases consist of a glycerol growth phase with a main goal of rapidly generating biomass, while repressing the AOX1 promoter in the absence of methanol. After the initial amount of glycerol in medium has been consumed the glycerol growth phase is then continued with a fed phase. This fed phase can be used to generate further biomass or for feed limitation to prepare the cells for methanol induction. The cells can be prepared for induction phase through feeding either with a growth limiting rate of glycerol feed or with a mixed feed containing both methanol and glycerol.

The glycerol growth phase allows reaching a desirable cell density without an increased risk of accumulating toxic amounts of metabolic products such as formaldehyde which is only produced in methanol phase. The risk of accumulating metabolic products occurring also in the glycerol phase such as ethanol are prevented further by the strong tendency of *P. pastoris* to physiologically favor respiratory rather than fermentative growth (21).

Methanol induction phase is reached after glycerol and other intermediate AOX1 repressing carbon sources have been consumed. The AOX1 gene is induced only by methanol and methanol will serve as the single carbon source. The strongly inducible AOX1 promoter triggers the production of the recombinant protease Tmprss2 only in the presence of methanol. *P. pastoris* is

flexible in whether the protein is produced intra- or extracellularly, but in the experiments conducted for this thesis work both Tmprss2-WT and Tmprss2-Mut cell lines produced Tmprss2 extracellularly. During the methanol expression phase methanol is initially oxidized by AOX to formaldehyde and hydrogen peroxide, the accumulation of these toxic intermediate products can be hazardous to the culture. Especially in the transition phase from glycerol to methanol. Furthermore, accumulation of methanol itself from feed is possible if instead of it *P. pastoris* is consuming carbon sources higher in its carbon source hierarchy that may be present in the transition phase or in low oxygen conditions. Therefore, real time measurement and adjustment of methanol and dissolved oxygen levels in methanol expression phase are crucial to avoid loss of culture. (25)

1.3 Flask cultivation

It was necessary to run initial tests on cultivation strategies in flasks prior to reactor experiments. Even though theoretically the ability to closer monitor and regulate cultivation conditions in a bioreactor should lead to higher cell densities and yields of desired product, several experiments could be run simultaneously for comparison in flasks. This offered a clear advantage on assessing iterative methanol induction cultivation mode variations as the only available reactor would have required to have successive runs taking up twice the amount of time used for the same experiments in flasks.

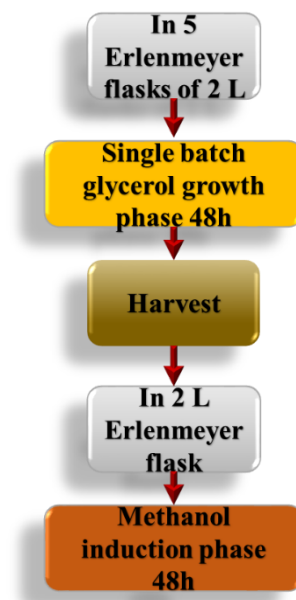


Figure 5. Cultivation phases of *P. pastoris* in flask cultivation. The glycerol growth phase resembles a single batch cultivation where all glycerol in the medium is consumed. In the harvest step the cells are separated from 5 L of BMGY-AF medium by centrifugation and resuspended in 1L of BMMY-AF medium effectively concentrating the culture 5:1. The methanol induction phase is a feed phase where the methanol level is replenished back up to 0.5% (V/V) every 24h to maintain induction.

Flask cultures were conducted in 1 L medium volume in baffled 2 L Erlenmeyer flasks placed in 28 °C Multitron incubation shaker at 130 rpm. The flasks were prone to producing a foam layer which was substantial enough to inhibit aeration. This issue was countered by addition of 5% (V/V) Struktol J 673 up to 0,1% (V/V) of the total medium volume.

Cultivation in flasks required concentrating cells 5:1 to reach higher OD for methanol induction phase. This was achieved by having a total of 5 L of preculture in BMGY distributed in five 2 L baffled Erlenmeyer flasks during glycerol phase and pooling the cells together by centrifugation and resuspending them in 1 L of BMMY for the methanol induction phase.

BMGY-AF and BMMY-AF media prepared according manual for *P. pastoris* expression (27) were used for flask cultures in their respective phases unless stated otherwise. The growth of *P. pastoris* in flasks was monitored every 24h. OD measurement as well as measuring the wet cell weight(WCW) during cell harvesting, while Tmprss2 production could only be detected and measured at the end of each production Cycle . Throughout the thesis work several patterns of feeding and repeating batches as well as single batches were run to test potential optimization strategies of Tmprss2 production. These tests included testing for effects of gradual methanol feeding in the beginning of induction phase instead of raising the levels directly to target levels. Another variable tested was increasing the target methanol level from 0.5% to 1%. Flask cultivation modes experimented with in 2 L flasks were single batch, iterative methanol induction and hybrid iterative methanol induction. In the hybrid mode, the glycerol growth phase took place in a 2 L Infors Labfors 3 reactor and the methanol induction phase in 2 L flasks.

1.4 Reactor cultivation

Considering large scale production, the media and cultivation condition requirements of *P. pastoris* are economical and make it a potential candidate for reactor scale cultivation and upscaling. In reactor, the full technical setup also allows for continuous carbon source feeding and monitoring of key cultivation parameters. The closely monitored and controlled environment creates potential for higher product yields and cell densities compared to flask cultivation. Reaching high cell densities also creates a need for reoptimization of cultivation conditions, but an optimized small scale reactor process can be considered representative of requirements for upscaled processes with only minor modifications. (21)

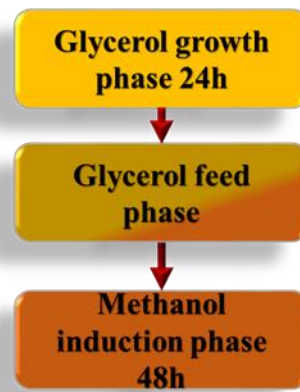


Figure 6. Common cultivation phases of *P. pastoris* in reactor cultivation. The glycerol growth phase resembles a single batch cultivation where all glycerol in the medium is consumed. The glycerol feed phase has many variations for feeding and duration, but in this thesis, it was implemented for approximately 6h with the goal to accumulate more biomass with maximal feeding and enhanced aeration. The methanol induction phase is also a fed phase where the methanol level is kept constant with an automated system to maintain induction.

Both Tmprss2-WT and -Tmprss2-Mut were cultivated in reactor only once during this bachelor thesis work, due to the limitation of available time. The cultivation strategy in reactor was based on hybrid cultivation tests conducted first in reactor for the glycerol feed phase and then divided into two cultures in flask cultivation mode for methanol induction phase. Iterative methanol induction cultivation mode was implemented in the same manner for both Tmprss2-WT and -Tmprss2-Mut for two iterations. This meant that each run (Cycle) produced approximately 2,5 litres of supernatant in total while this supernatant was purified and analyzed in two separate batches. Because of the fast degradation rate of Tmprss2 at 72 HPI significant degradation of Tmprss2 can already be recognized in the culture supernatant (12). The 48h length of methanol induction iteration was chosen to avoid extra loss of Tmprss2 as after harvest it still took 24h more to complete IMAC.

The amount of time required for batches was further reduced by shortening the glycerol growth phase by adding pure oxygen into the reactor airflow during exponential growth phase. This eliminated restriction on glycerol feed rate due to lack of aeration as there was no longer a risk of causing the pO₂ to decrease below desired 20% due to high metabolic rate during exponential growth.

In both Tmprss2-WT and -Tmprss2-Mut reactor cultivations AF was added far beyond the need for its primary purpose due to over sensitivity of the AF sensor. This resulted in the high concentrations of AF Struktol J673A which seem to increase recombinant protein expression and secretion in *P. pastoris* (26).

1.5 Iterative methanol induction cultivation mode

The common cultivation strategy divided in a glycerol and methanol phase leads to a process which is not producing Tmprss2 approximately half of the time. The glycerol phase serves only to produce biomass and Tmprss2 is only produced in methanol phase when AOX1 promoter is induced. When considering optimization of the process not only the peak production amounts should be considered, but also the time it takes to produce a batch of product. Iterative methanol induction can be used to cut down the time of production for consecutive batches by resuspending the already induced cells in fresh expression medium eliminating the need to repeat the glycerol growth phase. The expression can then be continued for another 48h producing an expression yield comparable to the first iteration in the second batch (12).

In this thesis work this iterative methanol induction cultivation mode was upscaled from flasks to a 2L reactor setup. In reactor scale the various probes needed to stay submerged and thus only 2/3 of reactor volume could be harvested between iterations. This meant that in reactor the iterative methanol induction cultivation mode was conducted as a repeated fed batch, in which the cells harvested between iterations were suspended in fresh reactor media without glycerol equal to the amount of expression media that was harvested. This fresh media was then fed back into the reactor and mixed with the 1/3 of old expression media while the reactor system automatically regulated the methanol levels back to the desired level keeping the cells induced.

While the fresh media and the feeds from the reactor cultivation setup provide everything required for maintaining the culture. In theory, the iterations could be repeated limitlessly without losing expression, creating a semi-continuous process. Increasing the successive amount of iterations only further increases the Tmprss2 production efficiency of the process as a whole, as the time required for the initial glycerol phases becomes a smaller fraction of the total process time with each iteration.

2 Aims

2.1 Production of mouse Tmprss2 in Pichia pastoris in sufficient amounts for crystallization experiments.

Production of Tmprss2 was measured qualitatively by mass-spectrometry, Western blot and SDS-PAGE to identify the product and its form. The produced amount of Tmprss2 was measured quantitatively by spectrophotometry. The aim was to generate 2 mg amounts of mouse Tmprss2 in active form for crystallization experiments.

2.2 Testing the viability of NiSepharose™ Excel material for direct immobilized metal ion affinity chromatography of Tmprss2.

IMAC was conducted in self-packed Bio-Rad column with NiSepharose™ Excel material as well as a premade HisTrap™ excel 5 mL column to test the two available excel IMAC products available from GE Healthcare GmbH. The success of IMAC was measured qualitatively by mass-spectrometry, Western blot and SDS-PAGE to determine whether Tmprss2 could be bound from the supernatant with this material. The amount of bound product was measured quantitatively by spectrophotometry. The viability of the material was assessed in the terms of stripping of the immobilized nickel ions and the achieved Tmprss2 binding compared to Ni-NTA His-Bind material.

2.3 Development of strategies for optimized cultivation of Pichia pastoris cell lines in 2 L scale using a bioreactor in iterative methanol induction cultivation mode.

The efficiency of the cultivation strategies was measured quantitatively in time (h), OD and WCW to find the effect of the improvements. The efficiency of methanol induction strategies was measured through protein production.

3 Materials

Materials used in this bachelor's thesis can be found listed in 9.3 Appendix 3. Materials list. The chemicals and reagents used were of analytical grade and when in solutions dissolved in Milli-Q water unless stated otherwise. Water used in experiments and solutions was Milli-Q water or autoclaved Milli-Q water when required.

All materials were provided by Helmholtz-Centre for Infection Research, Braunschweig, Germany and used in compliance with their requirements. All laboratory equipment was operated according to protocols provided by their respective manufacturers under parameters specified in individual topics in section 4. Methods.

4 Methods

4.1 Process flowsheets

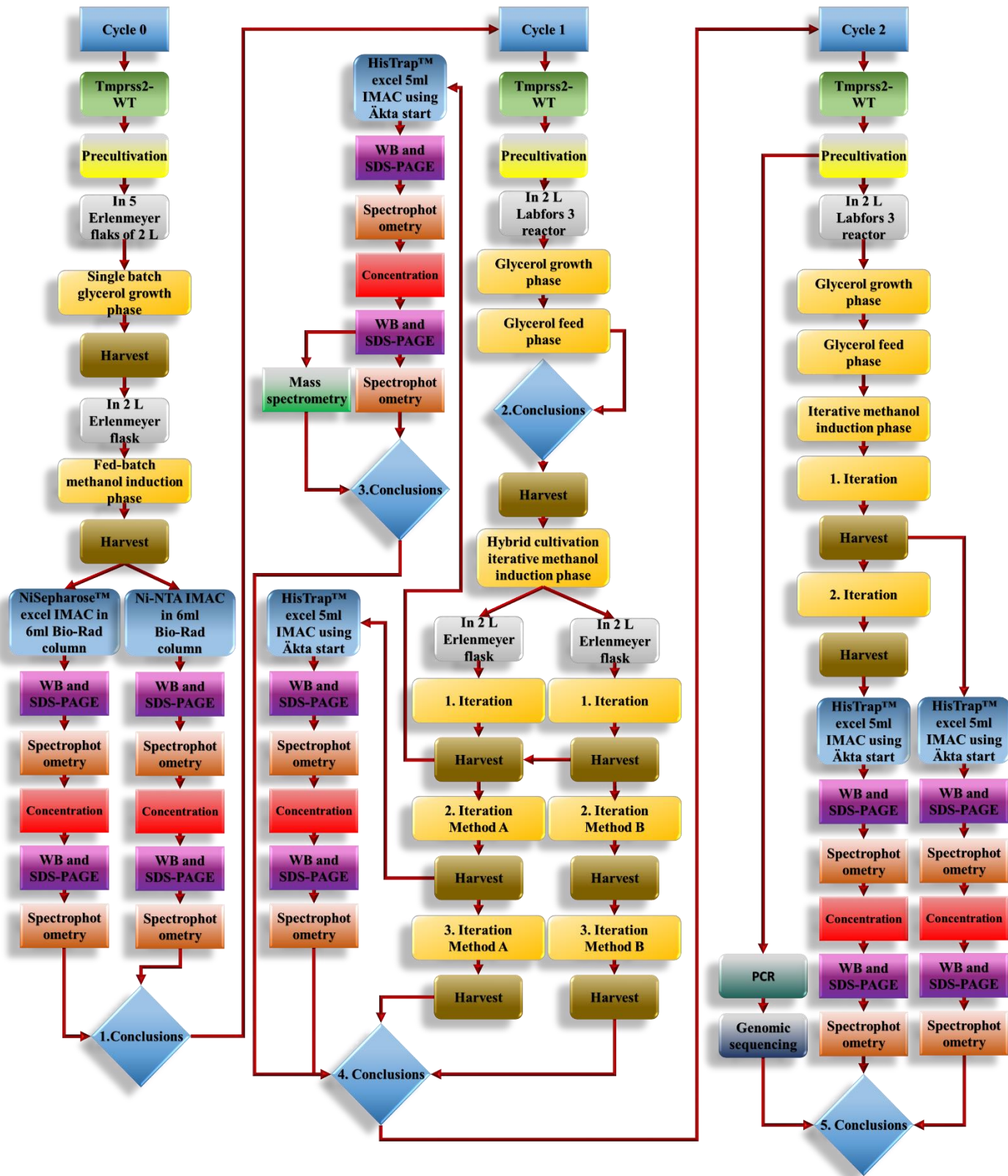


Figure 7. Process flowsheet for Cycles 0, 1 and 2;

- 1. Conclusions: Successful capture and concentration of Tmprss2 with NiSepharose™ excel see section 5.3.1 Cycle 0, Tmprss2-WT in 2 L Erlenmeyer flasks in fed-batch cultivation mode. Decision to upscale to 2 L Labfors 3 reactor and to perform IMAC with Histrap™ excel 5 mL column using Äkta start in Cycle 1;**
- 2. Conclusions: Hardware failure of connector between methanol sensor and feed pump. Since Automatic methanol feeding was not possible the culture was transferred into 2 L Erlenmeyer flasks and tests on two iteration strategies A and B were performed see section 4.2.2 Flask cultivation method;**
- 3. Conclusions: Iteration strategy A appeared more promising based on OD results and was chosen for purification see section 5.1.1 Cycle 1 optical density 595, Tmprss2-WT in 2 L Erlenmeyer flasks in hybrid iterative methanol induction cultivation modes A & B. IMAC with Histrap™ excel 5mL column using Äkta start was also shown to be successful see section 5.2 Protein production results and section 5.3.2 Cycle 1, Tmprss2-WT in 2 L Erlenmeyer flasks in hybrid iterative methanol induction cultivation mode. Tmprss2 band from C1.1 SDS-PAGE see Figure 23. was sent for mass spectrometry and confirmed to be Tmprss2 see 5.4.1 Cycle 1.1 concentrated total fraction;**
- 4. Conclusions: Method for iterative methanol induction cultivation mode in 2 L Labfors 3 reactor was devised based on Iteration method A see section 4.2.3 Reactor cultivation method.**
- 5. Conclusions: loss of expression or the produced Tmprss2 in reactor scale. Regarding cell mass and OD the method for iterative methanol induction cultivation mode was shown to be viable see section 5.1.2 Cycle 2 optical density 595 in methanol induction phase and was implemented also in Cycle 3. Cell line used in Cycles 0-2 was confirmed by C. Schinkowski at HZI to be Tmprss2-WT by PCR from the Cycle 2. precultivation sample.**

2. Conclusions: Anomaly in reactor detected see Figure 34, which led to suspicion of contamination. Microscopy performed on a reactor culture sample, but *only P. pastoris* cells detected. Reactor sample was plated and unknown bacterial contamination detected. Cultivation was aborted in accordance with safety requirements. Due to time limitation, there was no time for more reactor runs and a decision was made to make a flask cultivation of Tmprss2-Mut in Cycle 4.;

3. Conclusions: Major loss of Tmprss2 in concentration step was recognized see 5.2 Protein production results.

4.2 Cultivation methods

4.2.1 Preculture cultivation method

Cell line stock from -80°C storage was plated on YPD-plates by dilution streaking using an inoculation loop and incubated in 30°C for 48h to produce single colonies from the stock.

One of these single colonies from the first plate is then replated in the same manner and incubated in 30°C for 48h to produce multiple single colonies. The YPD-plate is then stored in 4°C to preserve the colonies for use in inoculations.

One of the storage single colonies was used per preculture to inoculate 200 mL of BMGY in 1 L baffled Erlenmeyer flask. The Erlenmeyer flask was then placed at constant 28°C in a shaking incubator at 130 rpm for 48h. The OD of the preculture was measured and the inoculation volume for reaching the required target inoculation OD of 1 was calculated.

4.2.2 Flask cultivation method



Figure 10. flasks before (top left) and after (bottom left) glycerol growth phase without AF; flask with AF in methanol induction phase (right).

In a standard fed batch flask cultivation also referred to as method A for hybrid cultivation, there were two phases. First a 48h glycerol growth phase in BMGY followed by a 48h expression phase in BMMY. The cultivation conditions for both were constant 28°C at 130 rpm in a shaking incubator. Sampling from the flasks as well as OD measurement was carried out every 24h as described in the methods sections 4.4.1 and 4.4.3 for these tasks. The glycerol phase was conducted in 5 baffled 2 L Erlenmeyer flasks. Each flask was filled with 1 L of BMGY-AF and inoculated to a starting OD of 1 from a fresh preculture. After 48h at the end of the glycerol feed phase the cells from each flask were harvested by centrifugation in 1 L sterile centrifuge bottles as described in the methods section 4.3.1. These cells were then resuspended in 1 L of BMMY-AF and transferred in to a baffled 2 L Erlenmeyer flask that was the placed back in the shaking incubator. Halfway through the expression phase at 24h 5mL of 100% methanol was fed in to the flask to raise methanol concentration back to 0.5%(V/V). After another 48h the end of expression phase was reached and the supernatant was harvested while the cells were discarded or were used for iterative batches. In hybrid cultivation during Cycle 1 the iterations were 72h instead of 48h to better follow the growth in method B. In method B only half of the culture volume was harvested and the culture was replenished back up to its original volume with only fresh BMMY-AF without returning the harvested cells into the flask.

4.2.3 Reactor cultivation method

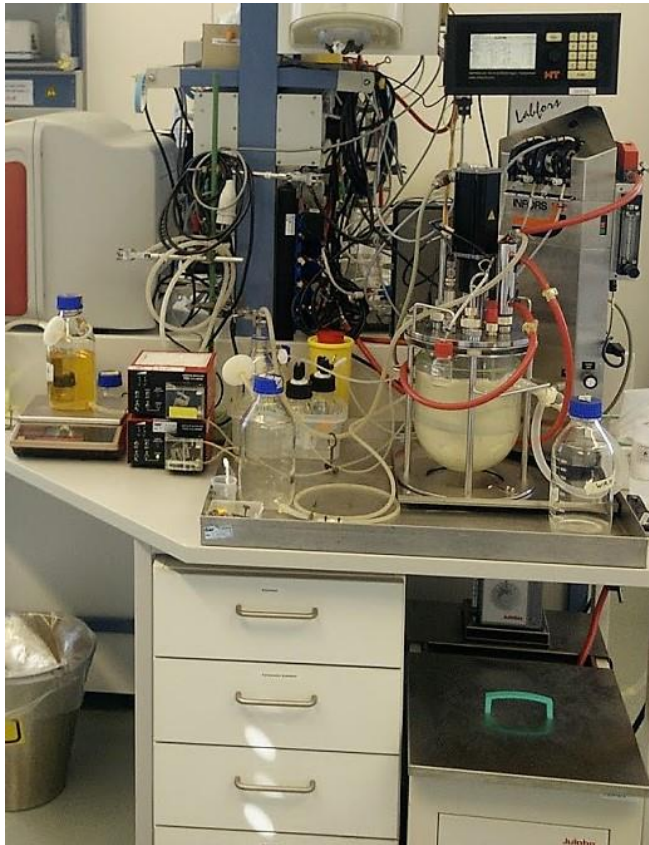


Figure 11. Reactor setup for 2 L Labfors 3 reactor in methanol induction phase.

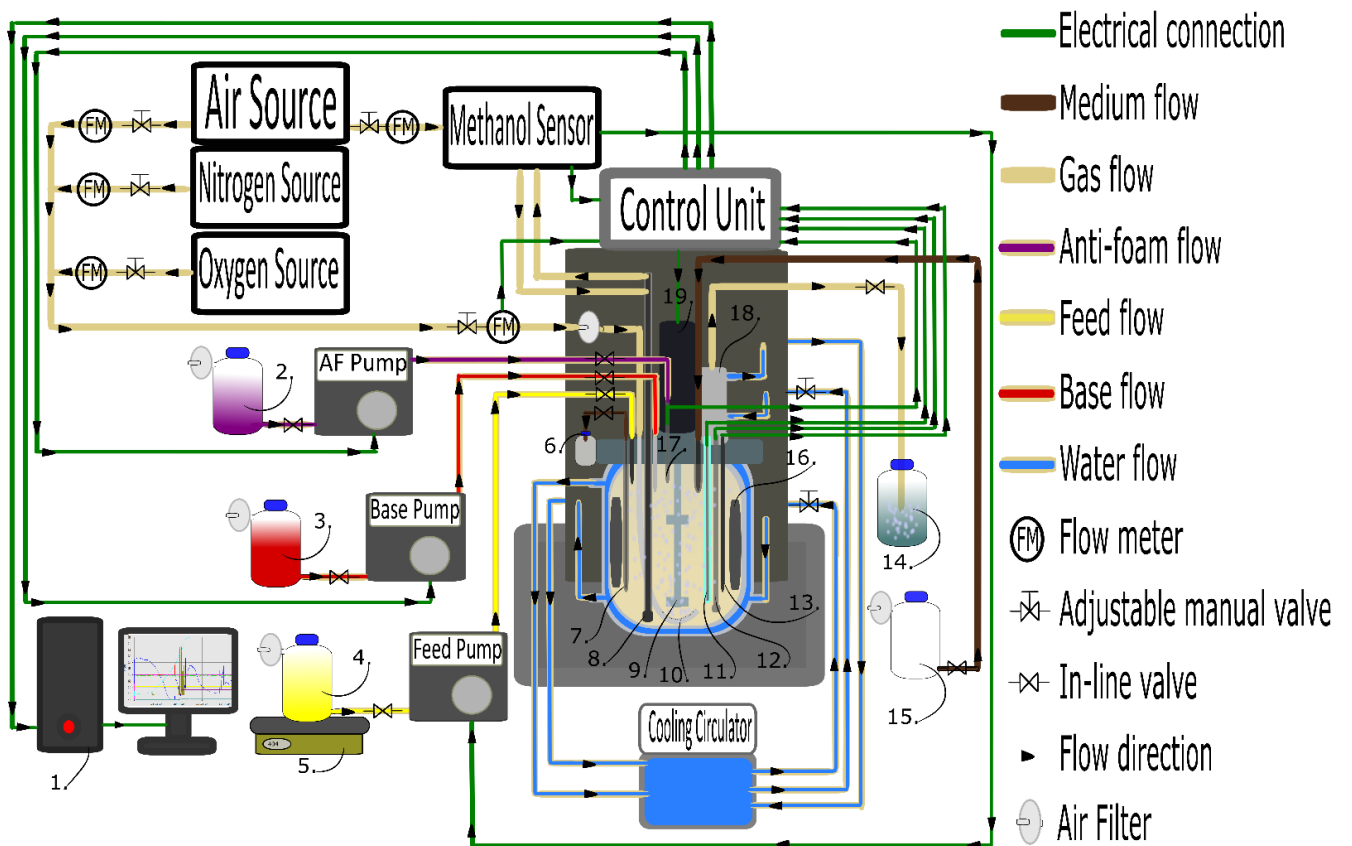


Figure 12. Schematic overview of reactor setup for 2 L Labfors 3 reactor;

1. PC for data collection running Iris software;
 2. 0,5 L Schott flask of 5% (V/V) Struktol J 673;
 3. 0,5 L Schott flask of 12.5% ammonium for pH adjustment;
 4. 1 L Schott flask of either methanol or glycerol feed depending on cultivation phase;
 5. Sartorius analytic BP 3100 S balance for monitoring feed weight;
 6. Sample collection outlet;
 7. Sample collection point;
 8. Raven biotech Inc. methanol sensor with separate air flow from the rest of the reactor;
 9. Overhead stirrer with 12 blades;
 10. Gas inlet inside the reactor;
 11. Mettler Toledo pH probe;
 12. Mettler Toledo pO₂ probe;
 13. Infors temperature probe;
 14. Exhaust gas outlet in 1 L Schott flask submerged in 1M sodium hydroxide;
 15. 1 L Schott flask for transferring media components and inoculum in reactor during filling phase;
 16. Stainless steel baffle;
 17. AF probe and inlet port;
 18. Water cooled cooler for exhaust gas;
 19. Motor for stirrer 400 V;
- “Control unit” Central control unit for Labfors 3 reactor;
- “Air Source” Dry instrument air from central HZI system;
- “Nitrogen Source” Dry nitrogen gas from central HZI system;
- “Oxygen Source” Dry oxygen gas from central HZI system;
- “Cooling Circulator” Julabo F34 set to 4°C automatically circulating cooling water to keep reactor at 28°C based on temperature probe signal and keeping exhaust gas cooler at 4°C;
- “AF Pump” Standard Infors pump belonging to Labfors 3 reactor system, automated pumping based on AF sensor signal;
- “Base Pump” Standard Infors pump belonging to Labfors 3 reactor system, automated pumping based on pH sensor signal;
- “Feed Pump” model 101U from Watson Marlow Fluid Technology Group. Feed pump is only controlled automatically by the methanol sensor during methanol feeding and is set manually for glycerol feeding;
- “Air Filter” Pall Gelman Acrodisc CR PTFE with 0.2 µm pore size from Sigma-Aldrich Co.

The reactor setup and media were based on the methods outlined in the book *Pichia protocols* (28) and the manual for 2 L cultivation in Labfors 3 reactor from Infors. The method for two iterations in a 2 L Infors Labfors 3 reactor in the iterative methanol induction cultivation mode could be implemented also for more successive iterations, but describing more than two would be redundant. Several steps for the first and second iterations had to take place simultaneously to ensure minimal stress, risk of contamination and time outside of the reactor for harvested cells.

Step 1. Preculture for reactor cultivation was prepared as outlined in section 4.2.1 Preculture cultivation method. Media, feeds and additives for controlling the cultivation conditions for the initial run and following iterations were prepared. Calibration liquids of pH 7 and 4 were used for calibrating the pH probe. Reactor was assembled and filled with base fraction of reactor media.

Step 2. The reactor containing reactor medium without hexametaphosphate fraction, glycerol feed and reactor medium without hexametaphosphate or glycerol for subsequent iteration steps were autoclaved.

Step 3 The reactor was assembled and the reactor run data collection started. Hexametaphosphate fraction of medium was added into the reactor. Stirring of the reactor was started.

Step 4. The reactor pH was adjusted to 6 with 12.5% ammonium through the reactor base feeding system. Reactor temperature was set to 28°C and temperature control with the cooling circulator started.

Step 5. The dissolved oxygen probe was calibrated by maintaining a 4 L/min flow of nitrogen and 1000 rpm stirring speed until the pO₂ value stabilized. This stabilized value was then set as 0% pO₂ reference value. The 100% pO₂ reference value was set by repeating the same process with air instead of nitrogen. 4 L/min flow of air was maintained as standard flow throughout the reactor run unless stated otherwise. Stirring speed was adjusted accordingly to maintain minimum 20% pO₂ and when only stirring with standard flow was not enough pure oxygen was added into the airflow. AF regulation was already started by the reactor adding some AF in the reactor medium to prevent complications from stirring speeds up to 1000 rpm as well as fill volume close to the reactor maximum due to feeding later in the process.

Step 6. OD of preculture was measured and inoculation volume for reaching inoculation OD of 1 was calculated. Test culture in reactor inoculated from the second preculture through the assigned port. Sampling and OD measurement were conducted as described in sections 4.4.2 Reactor sampling and 4.4.3 OD measurement.

Step 7. Glycerol growth phase was started by cultivating *P. pastoris* overnight in the reactor.

Step 8. The pO_2 was decreased close to 0% by the overnight cultivation. Stirring speed was increased and/or additional oxygen was introduced into the air flow to raise pO_2 above 20%.

Step 9. Sampling and OD measurement were conducted.

Step 10. Depletion of glycerol in the initial reactor media was observed as an pO_2 spike as *P. pastoris* ceased metabolizing it.

Step 11. Glycerol feed phase was started. Glycerol feeding rate was slowly increased along with the amount of additional oxygen in the air flow to achieve maximal feeding rate while keeping pO_2 above 20%. Glycerol feed phase was maintained for 5-6h.

Step 12. Sampling and OD measurement were conducted. The desirable OD in reactor for glycerol ending feed phase was $OD > 120$. When this target was reached, glycerol feeding was stopped.

Step 13. Methanol probe was calibrated by adding methanol feed slowly up to 0.5%(V/V) of the reactor volume in steps of 1-2%. The methanol sensor reading could stabilize in between the addition steps. Once a stable value for 0.5% was reached it was set as the set point for methanol feeding and the feed pump was set to automatic mode. This meant that as soon as the methanol concentration was detected by the methanol sensor to be below the set value it caused the feed pump to pump methanol feed into the reactor until the set point was reached. The calibration of the methanol probe and adding of methanol into the reactor also served as the transition phase into methanol induction marking the 0 HPI.

Step 14. After 24 HPI of the first 48h methanol induction iteration was started sampling and OD measurement were conducted as described in sections 4.4.2 Reactor sampling and 4.4.3 OD measurement. The sampling and OD measurement were repeated after 6h.

Step 15. After 48 HPI at the end of the first iteration. The reactor culture was sampled and OD was measured once again.

Step 16. 2/3 of reactor volume was harvested through the sampling port directly into 1L centrifuge bottles. The harvested fraction was purified as described in the section 4.2 Purification.

Step 17. The cells for first iteration were resuspended in an amount of reactor media without glycerol equal to the volume of culture that was harvested. This resuspended culture was then fed back into the reactor and the resulting mixture was sampled and its OD measured.

Step 18. Second iteration was carried out by repeating steps 14.- 16. with the exception of harvesting the full volume of the reactor in step 16..

Step 19. The cells from the final harvest were resuspended in water and autoclaved along with the reactor at the end of the cultivation process.

4.3 Purification methods

4.3.1 Centrifugation in harvesting phase

All centrifuging steps were carried out with a Sorvall RC 6+ centrifuge, but different rotors and centrifuge bottles were required for different centrifugation steps. Initially empty weights of 1 L and 500 mL centrifuge bottles as well as their lids were measured. 1 L centrifuge bottles were filled with media to similar weight with maximal difference of 0.02 g. Total weight of filled flasks was measured and recorded. The bottles were weighed after pouring out the supernatant to measure the weight of flasks containing the wet cell pellet from the previous centrifugation step between each round of centrifugation. This data along with the weight of filled flasks and empty weights of the bottles could then be used to calculate WCW. In first round of centrifugation cells were separated from supernatant by centrifuging at 6000 g for 10 min in 4 °C. The first centrifugation round at 6000 g was then repeated with a second set of fresh 1 L centrifuge bottles which were weighed and balanced just as the first set was. Resulting supernatant from the repeated first centrifugation round was poured into 500 mL centrifuge bottles, which were then weighed and balanced in the same manner as 1 L bottles in the previous step. The 500mL centrifuge bottles were centrifuged at 24500 g for 30 min at 4°C. The second centrifugation round at 24500 g was then repeated with a second set of fresh 500mL centrifuge bottles which were weighed and balanced just as the first set was. Resulting supernatant from the repeated second centrifugation round was then ready for filtration.

4.3.2 Filtration

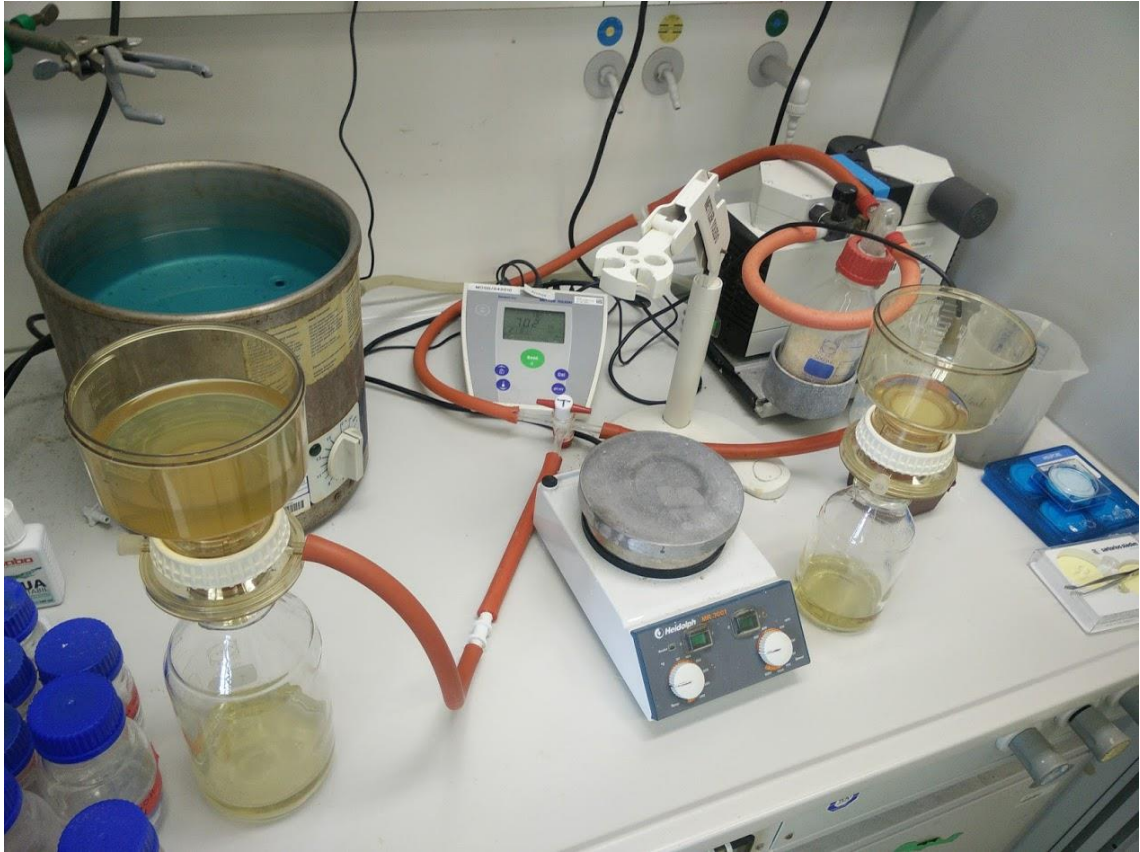


Figure 13. Setup for vacuum filtration with 2 NALGENE Reusable bottle top filter units.

Vacuum filtration

Prefiltration of culture supernatant was required before sterile filtration because without prefiltration approximately 10 Sartolab P20 pressure filtration units would have been required per litre of supernatant due to constant clogging up of the 0.2 μm pore size filters. After prefiltration by vacuum filtration only 1-2 Sartolab P20 pressure filtration units were used per sterile filtration of 1 L culture supernatant. Vacuum filtration was performed by simultaneously running 2 NALGENE Reusable bottle top filter units connected to 2 Schott flasks of 1 L each. Approximately 100mL of culture supernatant could be filtrated in vacuum filtration per 0.45 μm Cellulose Acetate Filter from Sartorius Stedim Biotech GmbH. Laboport series vacuum pump from KNF Neuberger Inc. was sufficient to create a vacuum for both filtration units. The vacuum filtration was preformed inside a Labcontrol fume hood from HESCO. Two units were used to half the time the culture supernatant had to be exposed to room temperature. The room temperature exposure was avoided to prevent Tmprss2 degradation.

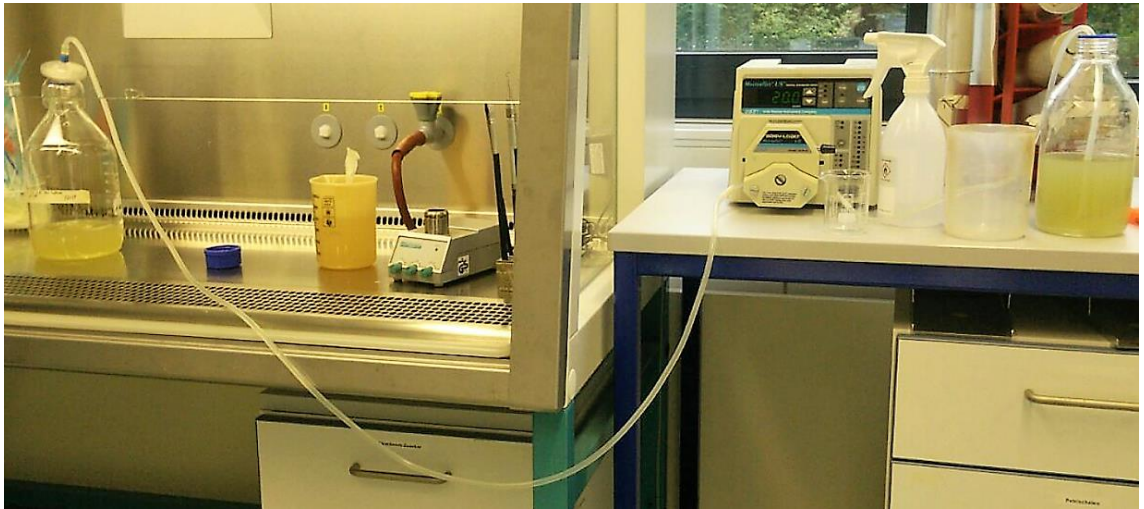


Figure 14. Setup for sterile filtration with Sartolab P20 pressure filtration units.

Sterile filtration

All media stock solutions for media components that were heat sensitive and could not be autoclaved were sterile filtrated prior to use. Culture supernatant was also sterile filtrated before IMAC to prevent microbial growth and remove leftover debris. Sartolab P20 pressure filtration units with 0.2 μm pore size were used for sterile filtration.

The material being filtrated was pumped using a Masterflex easy load L/S pump from Cole-Parmer Instrument Company through the filtration unit into a sterile Schott flask inside HERAsafe KS Biological Safety Cabinet from Thermo Fisher Scientific Inc.. The tubing for pumping was flushed with first with 100 mL 40% (V/V) ethanol followed by 200mL of Milli-Q water before and after filtration. Supernatant from cultivations was filtrated at 20 mL/min flowrate. Buffer solutions and media components were filtrated at 70 mL/min flowrate.

4.3.3 Immobilized metal ion affinity chromatography (IMAC)



Figure 14. Setup for Cycle 0 IMAC being washed with Milli-Q water.

IMAC in Cycle 0 was performed using separate hand packed Bio-Rad columns of 6mL NiSepharose™ excel and Ni-NTA His-Bind. Model 101U pump from Watson Marlow was used for pumping at 3 ml/min rate during loading and elution phases. This gave both the culture supernatant and elution buffers 2-minute residence time in the columns. The peak elution concentrations were identified by UV-absorption peaks using a Bio-Rad Laboratories Econo UV-Monitor. The complete peak fractions of 9-16 mL were then directly collected into 50 mL Corning® centrifuge tubes. Ni-NTA His-Bind required addition of Ni-ions directly into the culture supernatant prior to IMAC to prevent the column from getting completely stripped during loading. The nickel was added in the form of nickel sulphate up to 1 mM of the culture supernatant.



Figure 15. setup for IMAC with Äkta start in loading phase.

In Cycles 1-4 only NiSepharose™ excel material was used in the form of HisTrap™ excel 5mL prepacked column. The same column was used for all four cycles and cleaned per manufacturer instructions in between. Äkta start with automatic fractionation was used instead of the rudimentary setup in Cycle 0. Although peak fractions were still identified through UV-absorption also parameters like conductivity and pressure could be observed. Peak fractions were collected in 2mL fractions into glass test tubes using the automatic fractionation function of Äkta start. The exact parameters of the IMAC captures for Cycles 1-4 Can be seen in 9.2 Appendix 2. Äkta start IMAC logs.

4.3.4 Dialysis

Dialysis was performed in PBS using dry Spectra/Por™ dialysis tubes with a MWCO of 6000- 8000 kDa. The tubes were prepared for use by first saturating them in PBS buffer, this made them elastic and thus reduced the risk of the tubes being ripped during pipetting or by clamps used to seal the tube.

Fractions that had been identified by spectrophotometry and or WB to contain Tmprss2 were pooled together into the dialysis tube by pipetting.

Dialysis was performed overnight at 4 °C in 2 L of PBS in a 2L beaker. The PBS solution was continuously stirred with a magnetic stirrer at 100-200rpm.

4.3.5 Concentration

Heraeus MULTIFUGE 35-s from Thermo Fisher Scientific Inc. was used for centrifuging in all concentration processes at 3000-4000g depending on the cycle. Vivaspin tubes of varying sizes and with different MWCO were used for concentration in different cycles. All concentration steps were performed at 4°C.

Table 1. Cycle 0 concentration parameters

Cycle 0, 200mM imidazole buffer elution fraction from NiSepharose™excel capture	Value
Variant of Vivaspin tubes:	10 mL
MWCO:	30 kDa
Centrifugal force:	4 000 x g
Starting volume:	10 mL
Final volume:	0,5 mL
Concentration ratio:	20:1
Cycle 0, 500mM imidazole buffer elution fraction from NiSepharose™excel capture	Value
Variant of Vivaspin tubes:	10 mL
MWCO:	30 kDa
Centrifugal force:	4 000 x g
Starting volume:	16 mL
Final volume:	0,5 mL
Concentration ratio:	32:1
Cycle 0, 200mM imidazole buffer elution fraction from Ni-NTA His-Bind capture.	Value
Variant of Vivaspin tubes:	10 mL
MWCO:	30 kDa
Centrifugal force:	4 000 x g
Starting volume:	11 mL
Final volume:	0,5 mL
Concentration ratio:	22:1
Cycle 0, 500mM imidazole buffer elution fraction from Ni-NTA His-Bind capture.	Value
Variant of Vivaspin tubes:	10 mL
MWCO:	30 kDa
Centrifugal force:	4 000 x g
Starting volume:	16 mL
Final volume:	0,5 mL
Concentration ratio:	32:1

Table 2. Cycle 1-4 concentration parameters

Sample	Variant of Vivaspin tubes (mL)	MWCO (kDa)	Centrifugal force (x g)	Starting volume (mL)	Final volume (mL)	Concentration ratio
Cycle 1.1	2	10	3 000	8	0,1	80:1
Cycle 1.2	2	10	3 000	12	0,1	120:1
Cycle 2.1	N/A	N/A	N/A	N/A	N/A	N/A
Cycle 2.2	N/A	N/A	N/A	N/A	N/A	N/A
Cycle 3.1	2	5	3 000	8	1	8:1
Cycle 3.2	2	5	3 000	12	1	12:1
Cycle 4	2	5	3 000	10	1	10:1

4.4 Analytical methods

4.4.1 Sampling 2 L flasks

Sampling is conducted in HERAsafe KS Biological Safety Cabinet from Thermo Fisher Scientific Inc. using sterile plastic pipette tips. From each flask two samples are taken one for OD and one for storage. For each sample 1 mL is pipetted into a 1,5mL reaction cap. The OD sample is directly used for measurement. The sample for storage is first centrifuged with a Thermo Scientific Heraeus Fresco 17 centrifuge at 15 000 g for 10 minutes at 4°C. The liquid fraction is pipetted into another reaction cap while the pellet is preserved in the other. Both caps are then flash frozen with liquid nitrogen and stored in -20°C.

4.4.2 Reactor sampling

Sampling from the reactor always happens through the designated sampling port. By closing the air exhaust the incoming airflow creates pressure in the reactor. This pressure then pushes contents of the reactor out through the sampling port. First a waste sample is taken to flush the tubing of the sampling port, thereafter the actual sample is taken into a Corning® 15 mL centrifuge tube. From this sample a separate storage sample is taken by pipetting 1 mL into a 1,5mL reaction cap while the rest can be used for OD measurement. The storage sample is centrifuged at 15000 g for 10 minutes. The liquid fraction is pipetted into another reaction cap while the pellet is preserved in the other. Both caps are then frozen with liquid nitrogen and stored in -20°C.

4.4.3 Optical density 595 measurement

Fresh media of the corresponding type was used as blank for dilutions lower than 1:100, water was used for higher dilution ratios. The higher dilutions for example 1:1000 were conducted in dilution series in 10mL volume using Corning® 15 mL centrifuge tubes and vortexed between dilution steps. 1 mL disposable cuvettes were used for both samples and blanks. WPA CO 8000 Biowave Cell Density Meter set for 595 nm wavelength was used per manufacturer protocol for all OD measurements. Photometric Accuracy +/- 0.02A at 1A. Photometric Range -0.3 to 1.99A.

4.4.4 Electrophoresis

Most equipment for electrophoresis was from Bio-Rad and was operated in accordance with the methods supplied by the manufacturer. The buffer solutions were not premade but prepared at HZI laboratories per Bio-Rad recipes.

SDS-PAGE

Gel: 12% 10-well Bio-Rad Laboratories Mini-PROTEAN precast gel

Load amount: 20 µL

Sample: 15 µL

4xLoading buffer: 5 µL

Voltage: 140 V

Runtime :1h

Marker protein: Precision Plus Protein™ All Blue Prestained Protein

Coomassie staining solution for protein gels: Instant Blue

Western blot

Trans-Blot® Turbo™ Transfer System

Turbo mode Mini gels: 25 V/1,3A, 7 min, used for Cycles 0, 1. and 2.

Mixed mode Mini gels: 25 V/1,3A, 5 min, used for Cycles 3. and 4.

Membrane: Immobilon-P polyvinylidene difluoride (PVDF) from Merck

Primary anti-body: Mouse α-Flag

Secondary anti-body: Anti-Mouse IgG (H+L), Ap Conjugate

Colour Development Substrate: BCIP/NBT

4.4.5 Spectrophotometry

NanoDrop 2000/2000c Spectrophotometer from Thermo Fisher Scientific was used for measurements per instructions provided in NanoDrop 2000/2000c V1.0 User Manual by the manufacturer.

Parameters for measurement:

Mode: Purified BSA pedestal

Sample size: 1 μ L

Sample type: A280

Lower detection limit: 0.10 mg/mL

Upper detection limit: 400 mg/mL

Typical error: 0.10 - 10 mg/mL: + 0.10mg/mL; >10mg/mL: + 2%

4.4.6 Mass spectrometry

Mass spectrometry was performed by the research group Cellular Proteomics (CPRO) in HZI. The type of mass spectrometry used was MALDI-TOF (Matrix Assisted Laser Desorption Ionization – Time-Of-Flight). Sample bands were cut out from dried SDS-PAGE gels with a scalpel and stored in 1.5 mL Eppendorf tubes in 4°C prior to mass spectrometry.

4.5 Sterilization and preservation methods

4.5.1 Sterilization

The Labfors 3 reactor was autoclaved at 2 Bar at minimum 121°C for 75 minutes.

Non-heat sensitive stock solutions were sterilized by autoclaving at 2 Bar at minimum 121°C for 20 minutes.

Sterile filtration of media stock solutions which were heat sensitive as well as culture supernatant is described in section 4.3.2 Filtration.

4.5.2 Preservation

Filtrated culture supernatant was preserved by adding 0,1% (V/V) Sodium azide to prevent microbial growth. The produced Tmprss2 in the supernatant was preserved with cOmplete Mini, EDTA free Protease inhibitor cocktail tablets from Roche Diagnostics GmbH. 1 tablet was used per batch of produced supernatant which were in the range of 500 – 1400 ml.

Shock freezing by submerging liquid nitrogen was used to preserve cell and supernatant samples.

Cold storage devices for storage options in 4°C, -20°C and -80°C are listed in the materials list. -80°C storage was only used for master cell lines of Tmprss2-WT and Tmprss2-Mut while -20°C was used for flash frozen cell and supernatant samples. Stock solutions and samples that did not contain active cells could be stored in 4°C.

All cold storage devices were connected to HZI backup power supply system and would continue to operate on generator power during a power outage.

5 Results

5.1 *Optical density 595 measurement results*

5.1.1 Cycle 1 optical density 595, Tmprss2-WT in 2 L Erlenmeyer flasks in hybrid iterative methanol induction cultivation modes A & B

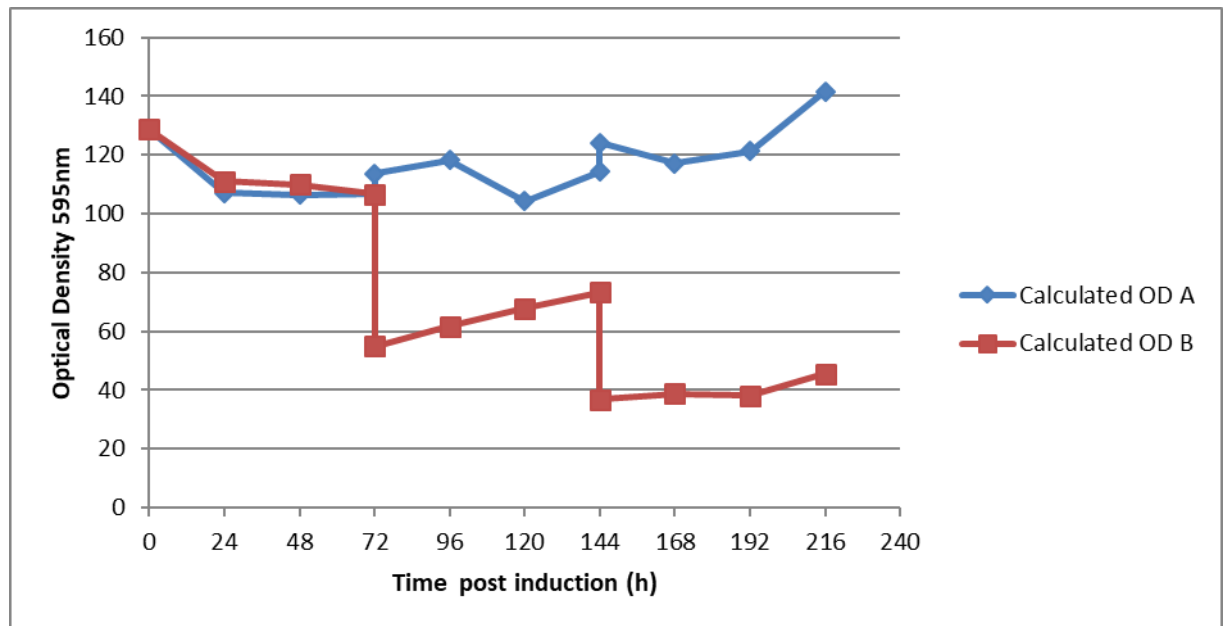


Figure 16. OD_{595} of iteration methods A and B over time; in method A full culture volume was harvested every 72h and the cultivation continued by resuspending the cells in fresh BMMY; in method B only half of the culture volume was harvested every 72h and the culture was replenished back up to its original volume with only fresh BMMY without returning the harvested cells into the flask.

Based on the growth kinetics for 3 iterations seen in Figure 16. the iteration method A was chosen as the more potential method. *P. pastoris* cultivated with method B could not regenerate its cell density between harvests at 72, 144 and 216 HPI. The A method showed no signs of decreasing cell density due to extra stress from three iterations.

5.1.2 Cycle 2 optical density 595 in methanol induction phase

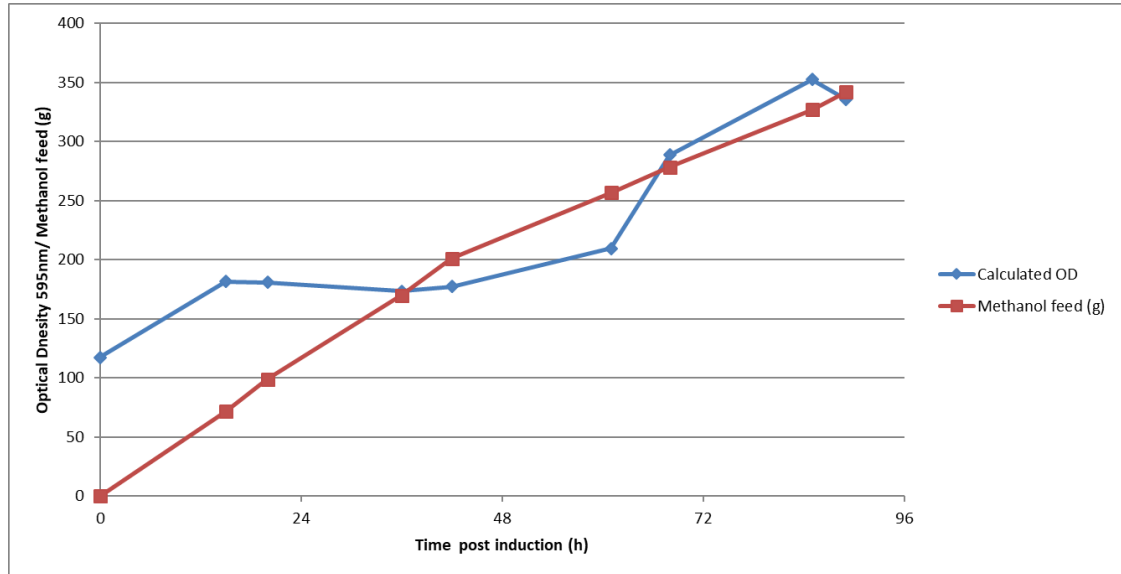


Figure 17. Cycle 2 OD₅₉₅ over time in relation to methanol feed in methanol induction phase, Tmprss2-WT in 2 L Labfors 3 reactor in iterative methanol induction cultivation mode

As can be seen the Tmprss2-WT in reactor cultivation appears to continue growth after iteration although there is a short lag phase. Unlike the growth curve for method A in Figure 16 the curve in Figure 17 doesn't enter stagnation, but reached OD₅₉₅ of 350 during two iterations. Instead of merely maintaining the reached cell density the Tmprss2-WT keeps growing in iterative methanol cultivation mode. The reason this was not observed in flasks is likely limitation by the methanol feed. In reactor Tmprss2-WT had automated constant methanol feed allowing it to continue growing even in higher OD.

5.1.3 Cycle 3 optical density 595 in relation to methanol feed, Tmprss2-Mut in 2 L Labfors 3 reactor in iterative methanol induction cultivation mode

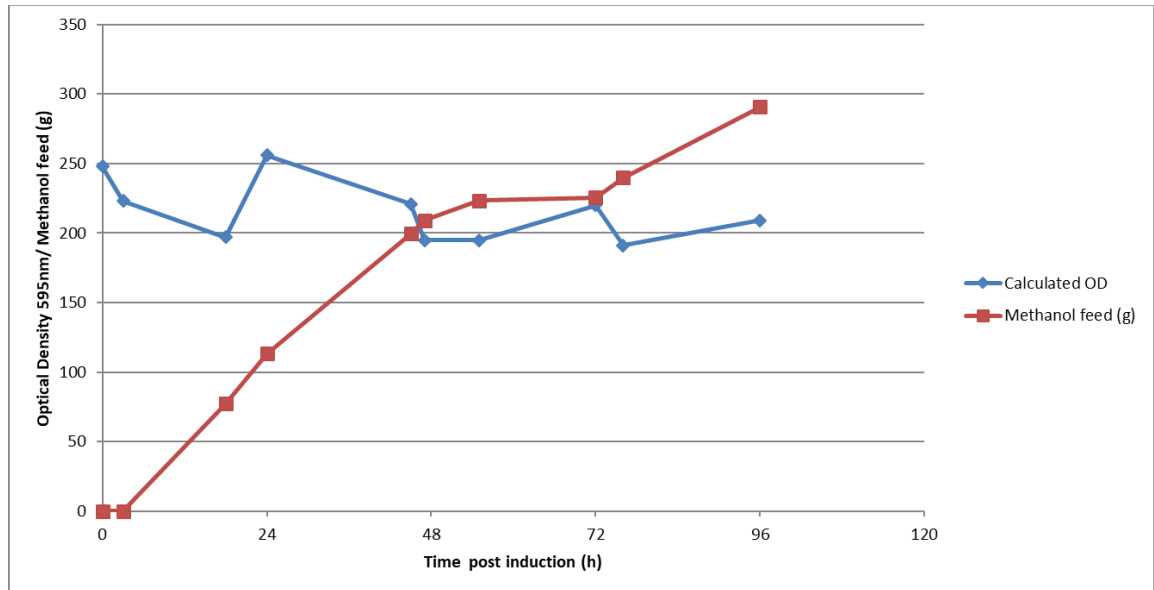


Figure 18. Cycle 3 OD₅₉₅ over time in relation to methanol feed in methanol induction phase, Tmprss2-Mut in 2 L Labfors 3 reactor in iterative methanol induction cultivation mode.

When comparing figures 17. and 18. clear differences between growth kinetics can be observed. Tmprss2-WT continues growth while the Tmprss2-Mut stagnates while still consuming methanol at a similar rate to Tmprss2-WT. This could possibly be explained by the Tmprss2-Mut being a Mut^s strain. Similar behaviour in iterative methanol induction by the same cell line was seen in (12).

5.2 Wet cell weight measurement results

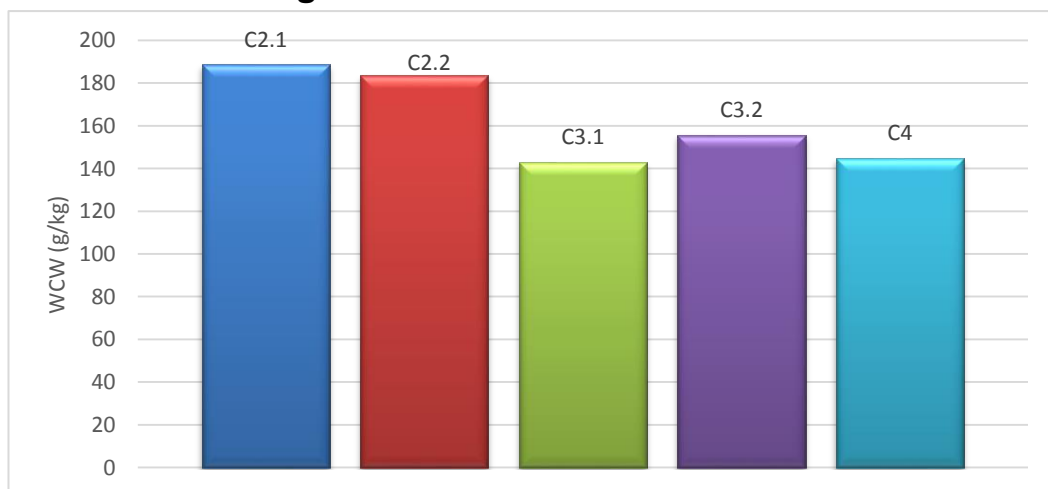


Figure 19. WCW in Cycles 2.1, 2.2, 3.1, 3.1 and 4 calculated from the cell pellet and medium weights from harvesting step.

The values in Figure 19 show no considerable reduction in WCW between iterations for either of the reactor runs in Cycle 2 or Cycle 3. The WCW for Cycle 4 being comparable with those for Cycles 2 and 3 demonstrates the comparability of flask and reactor experiments in these cell densities.

5.2 Protein production results

Table 3. Results for Tmprss2 production by *P. pastoris* measured with spectrophotometry. Values in parenthesis were consistently measured, but below the lower detection limit defined by the manufacturer.

Cycle	Sample	Calculated Tmprss2 yield (mg)	Tmprss2 concentration (mg/ml)	Blank
C0	20:1 Concentrated 200mM imidazole buffer elution fraction from NiSephacel™ excel capture	0,08	0,16	200 mM imidazole buffer
C0	32:1 Concentrated 500mM imidazole buffer elution fraction from NiSephacel™ excel capture	N/A	Below lower detection limit	500 mM imidazole buffer
C0	22:1 Concentrated 200mM imidazole buffer elution fraction from Ni-NTA His-Bind capture	N/A (0,05)	Below lower detection limit (0,09)	200 mM imidazole buffer
C0	32:1 Concentrated 500mM imidazole buffer elution fraction from Ni-NTA His-Bind capture	N/A	Below lower detection limit	500 mM imidazole buffer
C1.1	80:1 concentrated total fraction	N/A (0,04)	Below lower detection limit (0,09)	200 mM imidazole buffer
C1.2	120:1 concentrated total fraction	N/A	Below lower detection limit	200 mM imidazole buffer
C2.1	First iteration total fraction	N/A	Below lower detection limit	N/A
C2.2	Second iteration total fraction	N/A	Below lower detection limit	N/A
C3.1	8:1 concentrated first iteration dialyzed total fraction	1,42	1,42	PBS
C3.2	8:1 concentrated second iteration dialyzed total fraction	1,95	1,95	PBS
C4	unconcentrated dialyzed total fraction	4,78	0,48	PBS
C4	10:1 concentrated dialyzed total fraction	0,96	0,96	PBS

The protein production varied greatly between different Cycles and in general Tmprss2-Mut appeared to produce more Tmprss2 than the WT *P. pastoris* as was already observed by C. Schinkowski in (12). In previous experiments 0.8 mg/L yields of Tmprss2 D343N was achieved by Tmprss2-Mut and 80 µg/L of Tmprss2 by Tmprss-WT in flask cultures with similar IMAC (12). Based on the amount of Tmprss2 measured in individual elution fractions and unexpectedly low concentrations achieved in total concentrated fractions it was suspected that Tmprss2 was lost in the concentration step. This was finally demonstrated in Cycle 4 where the concentration of total dialyzed total fraction was measured before and after concentration. The concentration ratio was 10:1, but only increased the Tmprss2 concentration by roughly 2:1. The precipitate formed during the concentration was resuspended in 1 mL PBS and measured with NanoDrop and analysed with SDS-PAGE and WB. The resuspended precipitate was below measurement range on NanoDrop. The total concentrated fraction from Cycle 3.1 would be sufficient for crystallization by itself but the iterative methanol induction cultivation mode would allow for pooling the yields of consequent iterations together for even higher concentrations. This would be especially beneficial for performing crystallization with the processed form Tmprss2 produced dominantly by the Tmprss-WT.

5.3 SDS-PAGE and Western blot results

5.3.1 Cycle 0, Tmprss2-WT in 2 L Erlenmeyer flasks in fed-batch cultivation mode.

Western blot

Protease Tmprss2

Experiment: Cycle 0

Cell-line: P. pastoris Wild type jackpot clone KM71H-pPICZ α -Tmprss2-WTopt o20

Media: BMMY-AF

HPI = 48 h

Experimentator: Tli17

western blot (25 V/1,3A, 7 min)

1. antibody: Mouse α -Flag

1:4000 in TBST

Sigma Aldrich

2. antibody: Anti-Mouse IgG (H+L) AP

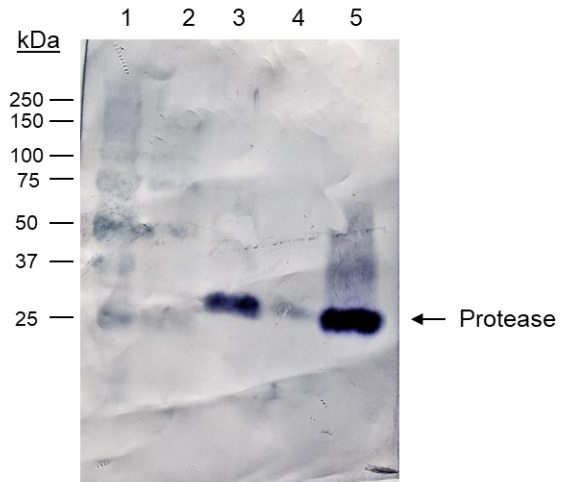
1: 7500 in TBST

Promega Corporation

development: BCIP/NBT

Size of protease: 30/45 kDa

Secreted protease



Lane	Sample	Amount
1	Marker (Precision Plus Protein™ All Blue Prestained Protein)	20 μ l
2	Concentrated elution fraction 5 from Ni-NTA capture (Elution with 500 mM imidazole buffer)	20 μ l
3	Concentrated elution fraction 3 from Ni-NTA capture (Elution with 200 mM imidazole buffer)	20 μ l
4	Concentrated elution fraction 5 from excel capture (Elution with 500 mM imidazole buffer)	20 μ l
5	Concentrated elution fraction 3 from excel capture (Elution with 200 mM imidazole buffer)	20 μ l

Figure 20. Western blot detection of concentrated Tmprss2-WT fractions purified with IMAC from Cycle 0.

In Cycle 0 the individual elution fractions were too diluted to give a signal on WB or SDS-PAGE or to be measured by spectrophotometry. After intensive concentration of 20:1 – 32:1 the peak fractions for 200 mM and 500mM buffers became visible on WB.

Both materials used for IMAC showed positive results for Tmprss2 production and capture from supernatant. The signal from NiSepharose™ excel was not only demonstrated to be suitable for Tmprss2 IMAC, but even had a stronger signal on the WB than Ni-NTA in this experiment. It could also be noted that most of the bound Tmprss2 was eluted already during the 200 mM imidazole elution. Tprss2-WT producing more of the processed variant of Tmprss2 was the expected result already observed in (12), but it was demonstrated once again with both IMAC materials showing bands only at approximately 30kDA.

5.3.2 Cycle 1, Tmprss2-WT in 2 L Erlenmeyer flasks in hybrid iterative methanol induction cultivation mode

Western blot

Protease Tmprss2

Experiment: Cycle 1.1

Cell-line: P. pastoris Wild type jackpot clone KM71H-pPICZ α -Tmprss2-WTopt o20

Media: BMMY-AF

HPI = 48 h

Experimentator: Tli17

western blot (25 V/1,3A, 7 min)

1. antibody: Mouse α -Flag

1:4000 in TBST

Sigma-Aldrich

2. antibody: Anti-Mouse IgG (H+L) AP

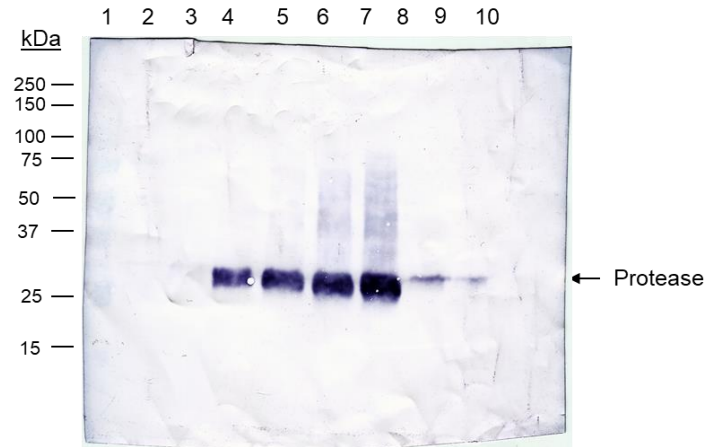
1:7500 in TBST

Promega Corporation

development: BCIP/NBT

Size of protein: 30/45 kDa

Secreted protease



Lane	Sample	Amount
1	Cycle 1.1 supernatant pre-capture	20 μ l
2	Fraction 2(Elution with 5 mM imidazole buffer)	20 μ l
3	Fraction 3 (Elution with 5 mM imidazole buffer)	20 μ l
4	Fraction 7 (Elution with 200 mM imidazole buffer)	20 μ l
5	Fraction 8 (Elution with 200 mM imidazole buffer)	20 μ l
6	Fraction 9 (Elution with 200 mM imidazole buffer)	20 μ l
7	Fraction 10(Elution with 200 mM imidazole buffer)	20 μ l
8	Fraction 16 (Elution with 500 mM imidazole buffer)	20 μ l
9	Fraction 17(Elution with 500 mM imidazole buffer)	20 μ l
10	Marker (Precision Plus Protein™ All Blue Prestained Protein)	20 μ l

Figure 21. Western blot detection of Tmprss2-WT fractions purified with IMAC from Cycle 1.1.

In the first iteration of Cycle 1 Äkta start was used for automatic fractionation during IMAC and this resulted in more concentrated individual fractions visible on WB without concentration steps. The trend of having most of the Tmprss2 eluted already in the first elution after washing was repeated also in Cycle 1.1 with only weak signal from the 500 mM imidazole elution.

Western blot**Protease Tmprss2**

Experiment: Cycle 1.1

Cell-line: P. pastoris Wild type jackpot clone KM71H-pPICZ α -Tmprss2-WTopt o20

Media: BMMY-AF

HPI = 48 h

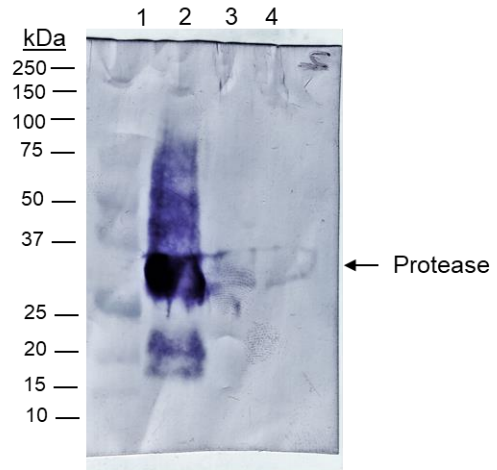
Experimentator: Tli17

western blot (25 V/1,3A, 7 min)

1. antibody: Mouse α -Flag
1:4000 in TBST
Sigma-Aldrich

2. antibody: Anti-Mouse IgG (H+L) AP
1:7500 in TBST
Promega Corporation

development: BCIP/NBT
Size of protease: 30/45 kDa
Secreted protease



Lane	Sample	Amount
1	Marker (Precision Plus Protein™ All Blue Prestained Protein)	20 μ l
2	Cycle 1.1 concentrated total fraction	20 μ l
3	Cycle 1.2 Fraction 13 (Elution with 500 mM imidazole buffer)	20 μ l
4	Cycle 1.2 Fraction 14 (Elution with 500 mM imidazole buffer)	20 μ l

Figure 22. Western blot detection of concentrated Tmprss2-WT fraction purified with IMAC from Cycle 1.1.

The four most potential fractions 7-10 from Cycle 1.1 were pooled together and successfully concentrated to produce a concentrated total fraction for the iteration. It is more likely that the imprints on lanes 3 and 4 are not actually from Cycle 1.2 elution fractions 13 and 14. It is far more likely that the signal seen there is caused by spillage or transfer from the strong signal on lane 2.

SDS-PAGE**Protease Tmprss2**

Experiment: Cycle 1.1

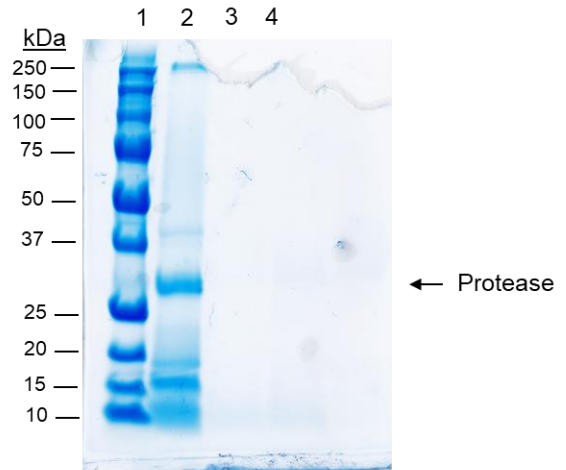
Cell-line: P. pastoris Wild type jackpot clone KM71H-pPICZ α -Tmprss2-WTopt o20

Media: BMMY

HPI = 48 h

Experimentator: Tli17

SDS-gel /12%
 Coomassie Blue
 Size of protease: 30 kDa/ 45kDa
 Secreted protease



Lane	Sample	Amount
1	Marker: Precision Plus Protein™ All Blue Prestained Protein	20 μ l
2	Cycle 1.1 concentrated total fraction	20 μ l
3	Cycle 1.2 Fraction 13 (Elution with 500 mM imidazole buffer)	20 μ l
4	Cycle 1.2 Fraction 14 (Elution with 500 mM imidazole buffer)	20 μ l

Figure 23. SDS detection of concentrated Tmprss2-WT fraction purified with IMAC from Cycle 1.1.

The total concentrated fraction from Cycle 1.1. was strong enough to produce a clear signal also on SDS-PAGE. The gel in figure 26. was dried and the band at 30 kDa on lane 2 was cut out and analyzed by mass spectrometry. Mass spectrometry confirmed the band to be mouse Tmprss2. This showed that the Tmprss2-WT cell line was producing the intended product and it was successfully captured by IMAC.

Western blot**Protease Tmprss2**

Experiment: Cycle 1.2

Cell-line: P. pastoris Wild type jackpot clone KM71H-pPICZ α -Tmprss2-WTopt o20

Media: BMMY-AF

HPI = 96 h

Experimentator: Tli17

western blot (25 V/1,3A, 7 min)1. antibody: Mouse α -Flag

1:4000 in TBST

Sigma-Aldrich

2. antibody: Anti-Mouse IgG (H+L) AP

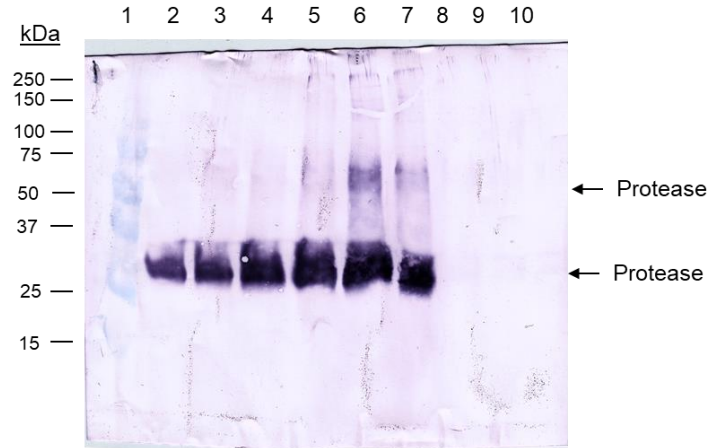
1:7500 in TBST

Promega Corporation

development: BCIP/NBT

Size of protease: 30/45 kDa

Secreted protease



Lane	Sample	Amount
1	Marker (Precision Plus Protein™ All Blue Prestained Protein)	20 μ l
2	Fraction 5 (Elution with 200 mM imidazole buffer)	20 μ l
3	Fraction 6 (Elution with 200 mM imidazole buffer)	20 μ l
4	Fraction 7 (Elution with 200 mM imidazole buffer)	20 μ l
5	Fraction 8 (Elution with 200 mM imidazole buffer)	20 μ l
6	Fraction 9 (Elution with 200 mM imidazole buffer)	20 μ l
7	Fraction 10(Elution with 200 mM imidazole buffer)	20 μ l
8	Fraction 14 (Elution with 500 mM imidazole buffer)	20 μ l
9	Fraction 15 (Elution with 500 mM imidazole buffer)	20 μ l
10	Marker (Precision Plus Protein™ All Blue Prestained Protein)	20 μ l

Figure 24. Western blot detection of Tmprss2-WT fractions purified with IMAC from Cycle 1.2.

The second iteration of Cycle 1 produced even stronger and more bands on WB than Cycle 1.1 most likely because of the increase of methanol feeding from 0.5 to 1%(V/V) during the second iteration. The concept and results of iterative methanol induction already outlined in (12) were replicated. The full length ectodomain of Tmprss2 was also visible in the strongest signals for the Cycle 1.2 fractions at 45 kDa especially in fraction 9 on lane 6. The total concentrated fraction for Cycle 1.2 produced a very weak signal on WB and was not detectable by spectrophotometry. While the individual fractions seen on lanes 2-7 seem very strong, it is highly likely that most of the Tmprss2 was lost during concentration step resulting in the lackluster total concentrated fraction.

5.3.3 Cycle 2, Tmprss2-WT in 2 L Labfors 3 reactor in iterative methanol induction cultivation mode

Loss of expression or failure to capture Tmprss2 was experienced in Cycle 2.

5.3.4 Cycle 3, Tmprss2-Mut in 2 L Labfors 3 reactor in iterative methanol induction cultivation mode

SDS-PAGE

Protease Tmprss2

Experiment: Cycle 3.1

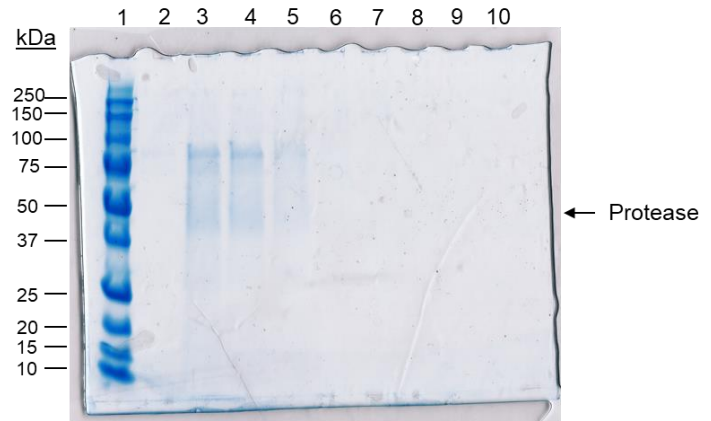
Cell-line: P. pastoris KM71H/pPICZ α -Tmprss2-D343Nopt clone o108

Media: BMMY-AF

HPI = 48 h

Experimentator: Tli17

SDS-gel /12%
Coomassie Blue
Size of protease: 30 kDa/ 45kDa
Secreted protease



Lane	Sample	Amount
1	Marker: Precision Plus Protein™ All Blue Prestained Protein	20 μ l
2	Fraction 2(Elution with 5 mM imidazole buffer)	20 μ l
3	Fraction 5 (Elution with 200 mM imidazole buffer)	20 μ l
4	Fraction 6 (Elution with 200 mM imidazole buffer)	20 μ l
5	Fraction 7 (Elution with 200 mM imidazole buffer)	20 μ l
6	Fraction 10 (Elution with 500 mM imidazole buffer)	20 μ l
7	Fraction 11(Elution with 500 mM imidazole buffer)	20 μ l
8	Fraction 12 (Elution with 500 mM imidazole buffer)	20 μ l
9	Fraction13 from cycle 3.1(Elution with 500 mM imidazole buffer)	20 μ l
10	Fraction 13 from cycle 2.2 (Elution with 200 mM imidazole buffer)	20 μ l

Figure 25. SDS-PAGE detection of Tmprss2-Mut fractions purified with IMAC from Cycle 3.1.

Western blot**Protease Tmprss2**

Experiment: Cycle 3.1

Cell-line: P. pastoris KM71H/pPICZ α -
Tmprss2-D343Nopt clone o108
mutS- strain

Media: Reactor medium

HPI = 48 h

Experimentator: Tli17

western blot (25 V/1,3A, 5 min)**1. antibody:** Mouse α -Flag

1:4000 in TBST

Sigma-Aldrich

2. antibody: Anti-Mouse IgG (H+L) AP

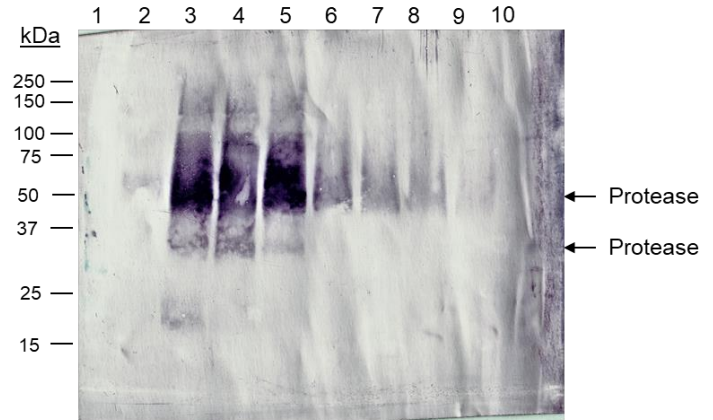
1:7500 in TBST

Promega Corporation

development: BCIP/NBT

Size of protease: 30/45 kDa

Secreted protease



Lane	Sample	Amount
1	Marker (Precision Plus Protein™ All Blue Prestained Protein)	20 μ l
2	Fraction 2(Elution with 5 mM imidazole)	20 μ l
3	Fraction 5 (Elution with 200 mM imidazole buffer)	20 μ l
4	Fraction 6 (Elution with 200 mM imidazole buffer)	20 μ l
5	Fraction 7 (Elution with 200 mM imidazole buffer)	20 μ l
6	Fraction 10 (Elution with 200 mM imidazole buffer)	20 μ l
7	Fraction 11(Elution with 200 mM imidazole buffer)	20 μ l
8	Fraction 12 (Elution with 500 mM imidazole buffer)	20 μ l
9	Fraction 13(Elution with 500 mM imidazole buffer)	20 μ l
10	Fraction 13 from cycle 2.2 (Elution with 200 mM imidazole)	20 μ l

Figure 26. Western blot detection of Tmprss2-Mut fractions purified with IMAC from Cycle 3.1.

The Cycle 3.1 SDS-PAGE and WB seen in Figures 25 and 26 show results expected from Tmprss2-Mut cultivation based on the results in (12). Higher Tmprss2 was yielded demonstrated in Figure 26 by individual unconcentrated elution fractions being visible on SDS-PAGE, which was never the case for Tmprss2-WT. Most of the produced Tmprss2 D343N appears to be in the 45 kDa uncleaved form although the weaker band of the processed form at 30 kDa is also visible.

Western blot**Protease Tmprss2**

Experiment: Cycle 3.2

Cell-line: *P. pastoris* KM71H/pPICZ α -
Tmprss2-D343Nopt clone o108
mutS- strain

Media: Reactor medium

HPI = 96 h

Experimentator: Tli17

western blot (25 V/1,3A, 5 min)1. antibody: Mouse α -Flag

1:4000 in TBST

Sigma-Aldrich

2. antibody: Anti-Mouse IgG (H+L) AP

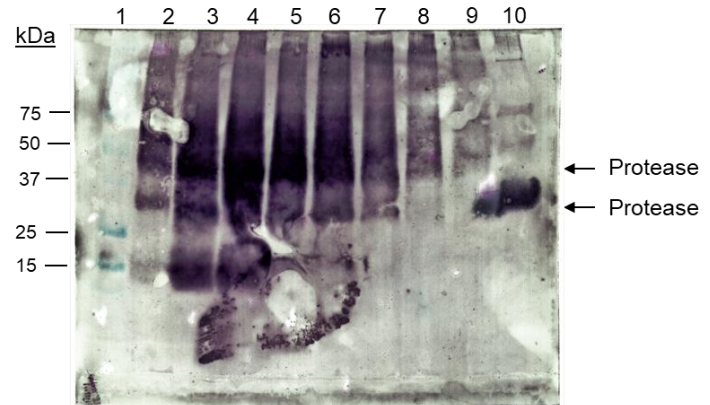
1:7500 in TBST

Promega Corporation

development: BCIP/NBT

Size of protease: 30/45 kDa

Secreted protease



Lane	Sample	Amount
1	Marker (Precision Plus Protein™ All Blue Prestained Protein)	20 μ l
2	Fraction 1 (Elution with 200 mM imidazole buffer)	20 μ l
3	Fraction 2 (Elution with 200 mM imidazole buffer)	20 μ l
4	Fraction 3 (Elution with 200 mM imidazole buffer)	20 μ l
5	Fraction 4 (Elution with 200 mM imidazole buffer)	20 μ l
6	Fraction 5 (Elution with 200 mM imidazole buffer)	20 μ l
7	Fraction 6 (Elution with 200 mM imidazole buffer)	20 μ l
8	Fraction 7 (Elution with 500 mM imidazole buffer)	20 μ l
9	Fraction 8 (Elution with 500 mM imidazole buffer)	20 μ l
10	C3.2 Supernatant post IMAC	20 μ l

Figure 27. Western blot detection of Tmprss2-Mut fractions purified with IMAC from Cycle 3.2.

In the second iteration of Cycle 3 the Tmprss2 WB signal was at its highest throughout the thesis work. There also appeared to be leftover Tmprss2 in the culture supernatant after IMAC detected by a strong signal on lane 10 in Figure 27. This supernatant sample was also run on a SDS-PAGE gel and the resulting band was sent to mass spectrometry for identification. The signal on lane 10 was identified as a *P. pastoris* cell wall protein and the similar molecular weight to Tmprss2 was merely a coincidence. Thus, the suspicion that there was a significant amount of Tmprss2 remaining in the supernatant after IMAC could be dismissed.

Western blot**Protease Tmprss2**

Experiment: Cycle 3 concentrations

Cell-line: P. pastoris KM71H/pPICZ α -
Tmprss2-D343Nopt clone o108
mutS- strain

Media: Reactor medium

HPI = 48/96 h

Experimentator: Tli17

western blot (25 V/1,3A, 5 min)**1. antibody:** Mouse α -Flag

1:4000 in TBST

Sigma-Aldrich

2. antibody: Anti-Mouse IgG (H+L) AP

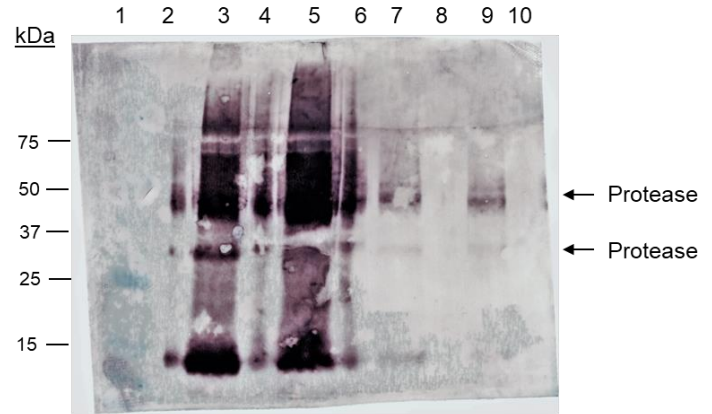
1:7500 in TBST

Promega Corporation

development: BCIP/NBT

Size of protease: 30/45 kDa

Secreted protease



Lane	Sample	Amount
1	Marker (Precision Plus Protein™ All Blue Prestained Protein)	20 μ l
2	X	X
3	Cycle 3.1 concentrated total fraction	20 μ l
4	X	X
5	Cycle 3.1 concentrated total fraction	20 μ l
6	X	X
7	Cycle 3.1 concentration precipitate dissolved in 1 ml PBS	20 μ l
8	X	X
9	Cycle 3.2 concentration precipitate dissolved in 1 ml PBS	20 μ l
10	X	X

Figure 28. Western blot detection of concentrated Tmprss2-Mut fractions purified with IMAC from Cycle 3.

The concentration of both Cycle 3.1 and 3.2 total fractions appears to have been equally successful. There only appeared to be trace amounts of Tmprss2 in the precipitate formed during the concentration seen on lanes seven and nine showing that the Tmprss2 was not being lost in to the precipitate during concentration step.

SDS-PAGE**Protease Tmprss2**

Experiment: Cycle 3 concentrations

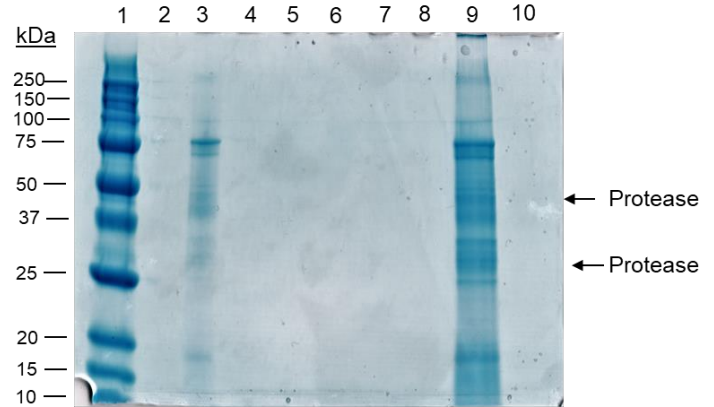
Cell-line: P. pastoris KM71H/pPICZ α -
Tmprss2-D343Nopt clone o108

Media: Reactor medium

HPI = 48/96 h

Experimentator: Tli17

SDS-gel /12%
Coomassie Blue
Size of protease: 30 kDa/ 45kDa
Secreted protease



Lane	Sample	Amount
1	Marker: Precision Plus Protein™ All Blue Prestained Protein	20 μ l
2	X	X
3	Cycle 3.1 concentrated total fraction	20 μ l
4	X	X
5	Cycle 3.2 concentrated total fraction	20 μ l
6	X	X
7	Cycle 3.1 concentration precipitate dissolved in 1 ml PBS	20 μ l
8	X	X
9	Cycle 3.2 concentration precipitate dissolved in 1 ml PBS	20 μ l
10	X	X

Figure 29. SDS-PAGE detection of concentrated Tmprss2-Mut fractions purified with IMAC from Cycle 3.

In Figure 29. the increase of contaminants from C3.1 to C3.2 can be observed. It also appears that separation of contaminants by precipitation during the concentration process was only successful in Cycle 3.2.

Western blot**Protease Tmprss2**

Experiment: Cycle 3.1 Autocatalytic cleavage and degradation experiment

Cell-line: WT- & Mut-P. Pastoris

Media: Reactor medium

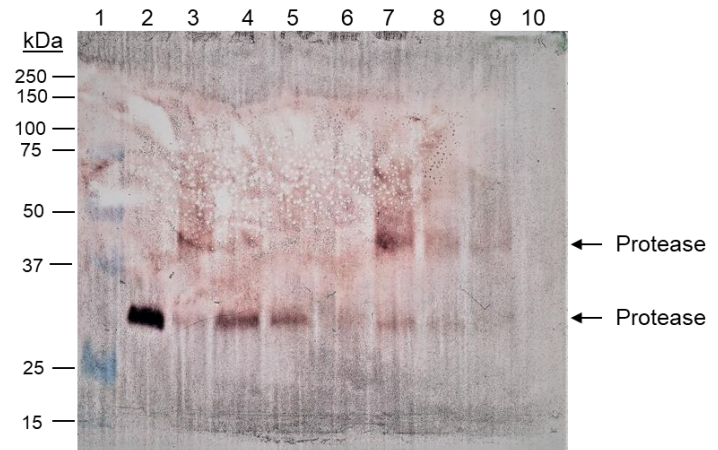
HPI = 48 h

Experimentator: Tli17

western blot (25 V/1,3A, 5 min)

1. **antibody:** Mouse α -Flag
1:4000 in TBST
Sigma-Aldrich
2. **antibody:** Anti-Mouse IgG (H+L) AP
1:7500 in TBST
Promega Corporation

Development: BCIP/NBT
Size of protease: 30/45 kDa
Secreted protease



Lane	Sample	Amount
1	Marker (Precision Plus Protein™ All Blue Prestained Protein)	15 μ l
2	Concentrated total fraction from Cycle 1.1	15 μ l
3	1 HPI Mixed Tmprss2-WT 1:100 (Concentrated fraction from C1.1) & Tmprss2-Mut 100:1 (Concentrated fraction from C3.1)	15 μ l
4	5 HPI Mixed Tmprss2-WT 1:100 (Concentrated fraction from C1.1) & Tmprss2-Mut 100:1 (Concentrated fraction from C3.1)	15 μ l
5	24 HPI Mixed Tmprss2-WT 1:100 (Concentrated fraction from C1.1) & Tmprss2-Mut 100:1 (Concentrated fraction from C3.1)	15 μ l
6	X	X
7	1HPI Tmprss2-Mut (Concentrated fraction from C3.1)	15 μ l
8	1HPI Tmprss2-Mut (Concentrated fraction from C3.1)	15 μ l
9	1HPI Tmprss2-Mut (Concentrated fraction from C3.1)	15 μ l
10	X	X

Figure 30. Western blot detection results of degradation and autocatalytic processing experiment by coinubation of C3.1 concentrated total fraction with 1:100 Cycle 1.1 concentrated total fraction.

It appears that adding only a fraction of the processed Tmprss2 to a higher amount of the unprocessed form of Tmprss2 and incubating them together at 37 °C autocatalyzes the unprocessed Tmprss2 D343N in a matter of hours. This can be observed by the change of composition in the mix of Tmprss2 and Tmprss2 D343N in lanes 3,4 and 5. Based on the change of intensity between the bands at 45 and 30 kDa it would appear that for mixed fractions the 30 kDa band increases in intensity before deteriorating. The control of only Tmprss2 D343N on lanes 7, 8 and 9 deteriorates at a steady rate without changes in its composition.

5.3.5 Cycle 4, Tmprss2-Mut in 2 L Erlenmeyer flasks in fed-batch cultivation mode

Western blot

Protease Tmprss2

Experiment: Cycle 4

Cell-line: P. pastoris KM71H/pPICZα-Tmprss2-D343Nopt clone o108 mutS- strain

Media: Reactor medium

HPI = 48 h

Experimentator: Tli17

western blot (25 V/1,3A, 5 min)

1. antibody: Mouse α-Flag

1:4000 in TBST

Sigma-Aldrich

2. antibody: Anti-Mouse IgG (H+L) AP

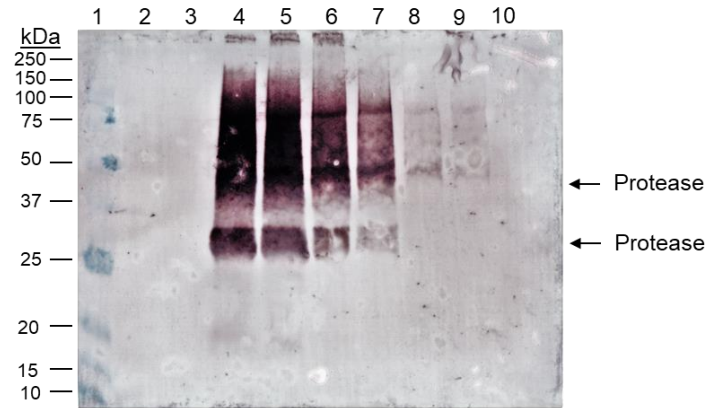
1:7500 in TBST

Promega Corporation

Development: BCIP/NBT

Size of protease: 30/45 kDa

Secreted protease



Lane	Sample	Amount
1	Marker (Precision Plus Protein™ All Blue Prestained Protein)	20 µl
2	Supernatant pre capture	20 µl
3	Fraction 3 (Elution with 5 mM imidazole buffer)	20 µl
4	Fraction 7 (Elution with 200 mM imidazole buffer)	20 µl
5	Fraction 8 (Elution with 200 mM imidazole buffer)	20 µl
6	Fraction 9 (Elution with 200 mM imidazole buffer)	20 µl
7	Fraction 10 (Elution with 500 mM imidazole buffer)	20 µl
8	Fraction 16 (Elution with 500 mM imidazole buffer)	20 µl
9	Fraction 17 (Elution with 500 mM imidazole buffer)	20 µl
10	Supernatant post capture	20 µl

Figure 31. Western blot detection of Tmprss2-Mut fractions purified with IMAC from Cycle 4.

In Cycle 4 very prominent bands of also the processed 30 kDa Tmprss2 were produced. The intensity of the bands is well in line with the high individual spectrophotometry results of Cycle 4 fractions. The fraction 7 on lane 4 in Figure 31. represents by itself 1,488 mg of Tmprss2 D343N in a 2 mL fraction. This is a prime example of how the western blot and spectrophotometry results had a strong correlation with the high and narrow peak in UV-absorption during IMAC seen in Appendix 9.2.8.

5.4 Mass spectrometry results

5.4.1 Cycle 1.1 concentrated total fraction

Reference sequence: Tmprss2-WT [Mus musculus]

(EAEAEFG)DSNCSTSEMECGSSGTCISSSLWCDGVAHCPNGEDENRCVRLY
 GQSFILQVYSSQRKAWYPVCQDDWSESYGRAACKDMGYKNNFYSSQGIPDQ
 SGATSFMKLVSSGNVDLYKKLYHSDSCSSRMVVSLRCIECGVRSVKRQSRI
 VGGLNASPGDWPWQVSLHVQGVHVCGGSIITPEWIVTAAHCVEEPLSSPRY
 WTAFAGILRQSLMFYGSRHQVEKVISHPNYDSK **TKNNDIALMK** LQTPLAFNDL
 VKPVCLPNPGMMLDLQECWISGWGATYEKGKTSVDLNAAMVPLIEPSKCNS
 KYIYNNLITPAMICAGFLQGSVDSCQGDSGGPLVTLKNGIWWLIGDTSWGSGC
 AKALRPGVYGNVTVFTDWIYQQMRANSENLYFQGACDYKDDDDKHHHHHHH
 H

The sequences in brackets are the remainders of the secretion signal that might still be attached to the protein. The WT-Tmprss2 sequence TKNNDIALMK is highlighted with red in the total sequence.

Result sequence:

Sample ID: 615 blue

>gi|74222080|dbj|BAE26858.1| unnamed protein product [Mus musculus]

MALNSGSPPGIGPCYENHGYQSEHICPPRPPVAPNGYNLYPAQYYPSPVPQY
 APRITTQASTSVIHHPKSSGALCTSKSKSLCLALALGTVLTGAAVAALLWR
 FWDSNCSTSEMECGSSGTCISSSLWCDGVAHCPNGEDENRCVRLYGQSFIL
 QVYSSQRKAWYPVCQDDWSESYGRAACKDMGYKNNFYSSQGIPDQSGATS
 FMKLVSSGNVDLYKKLYHSDSCSSRMVVSLRCIECGVRSVKRQSRI
 VGGLNASPGDWPWQVSLHVQGVHVCGGSIITPEWIVTAAHCVEEPLSSPRY
 WTAFAGILRQSLMFYGSRHQVEKVISHPNYDSK **TKNNDIALMK** LQTPLAFNDL
 VKPVCLPNPGMMLDLQECWISGWGATYEKGKTSVDLNAAMVPLIEPSKCNS
 KYIYNNLITPAMICAGFLQGSVDSCQGDSGGPLVTLKNGIWWLIGDTSWGSGC
 AKALRPGVYGNVTVFTDWIYKQMRANS

The WT-Tmprss2 sequence TKNNDIALMK highlighted with red in the total sequence, was also found in the result sequence.

Table 4. Theoretical molecular weights for the Tmprss2-WT sequence for an oxidized sample of the Tmprss2-WT. Values in red and blue were found in the spectra for the same sample seen in Figure 32.

Peptid-ID: 2.0					
no	acid	B [M+H] ⁺	B [M+2H] ²⁺	Y [M+H] ⁺	Y [M+2H] ²⁺
1	T	102,055	51,531	-	-
2	K	230,15	115,579	1062,561	531,784
3	N	344,193	172,6	934,466	467,737
4	N	458,236	229,622	820,423	410,715
5	D	573,263	287,135	706,38	353,694
6	I	686,347	343,677	591,353	296,18
7	A	757,384	379,196	478,269	239,638
8	L	870,468	435,738	407,232	204,12
9	M	1017,503	509,255	294,148	147,578
10	K	-	-	147,113	74,06
sequence:TKNNDIALMK					

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615bl. JVH

M1294C #6-30 RT: 0.157-0.848 AV: 25 NL: 1.31E5
 T: FTMS + p NSI Full ms2 582.90@hcd25.00 [100.00-1200.00]

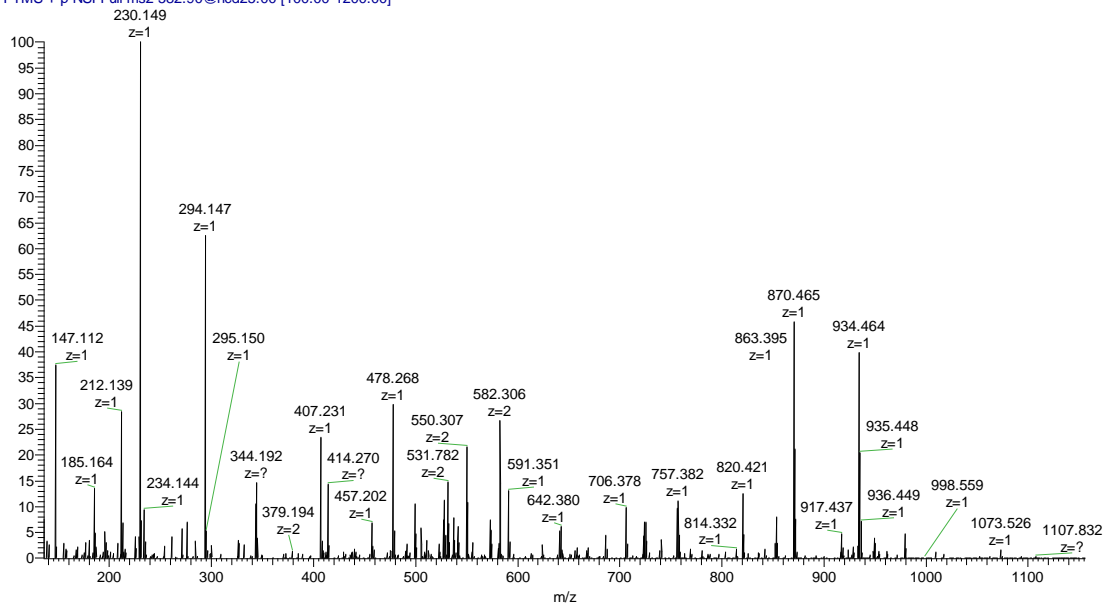


Figure 32. Spectra for the oxidised sample of the Tmprss2-WT sample.

Table 5 theoretical molecular weights for the Tmprss2-WT sequence for an unoxidized sample of the Tmprss2-WT.

Peptid-ID: 3.0					
no	acid	B [M+H] ⁺	B [M+2H] ²⁺	Y [M+H] ⁺	Y [M+2H] ²⁺
1	T	102,055	51,531	-	-
2	K	230,15	115,579	1046,566	523,787
3	N	344,193	172,6	918,471	459,739
4	N	458,236	229,622	804,428	402,718
5	D	573,263	287,135	690,385	345,696
6	I	686,347	343,677	575,359	288,183
7	A	757,384	379,196	462,274	231,641
8	L	870,468	435,738	391,237	196,122
9	M	1001,508	501,258	278,153	139,58
10	K	-	-	147,113	74,06
sequence :TKNNDIALMK					

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615bl. JVH

M1294B #30-32 RT: 0.860-0.919 AV: 3 NL: 2.93E4
T: FTMS + p NSI Full ms2 574.30@hcd28.00 [100.00-1200.00]

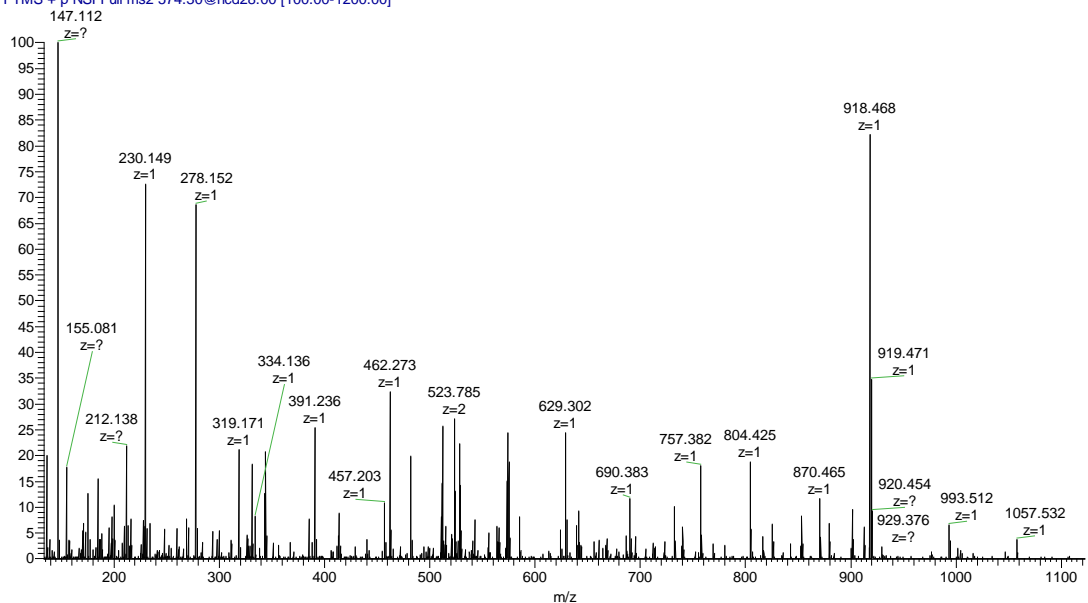


Figure 33. Spectra for the unoxidized sample of the Tmprss2-WT sample.

The suspected Tmprss2 band from Cycle 1.1 was confirmed by mass spectrometry to be Tmprss2. This confirmed that the cell line used was indeed Tmprss-WT. This was also an indicator for decent purity of the produced Tmprss2.

5.4.2 Cycle 3.1 Supernatant post IMAC

Reference sequence: Tmprss2-D343N [Mus musculus]

(EAEAEFG)DSNCSTSEMECGSSGTCISSSLWCDGVAHCPNGEDENRCVRLY
 GQSFILQVYSSQRKAWYPVCQDDWSESYGRAACKDMGYKNNFYSSQGIPDQ
 SGATSFMKLVSSGNVDLYKKLYHSDSCSSRMVVSLRCIECGVRSVKRQSRI
 VGGLNASPGDWPWQVSLHVQGVHVCGGSIITPEWIVTAAHCVEEPLSSPRY
 WTAFAGILRQSLMFYGSRHQVEKVISHPNYDSK **TKNNNIALMK**LQTPLAFNDL
 VKPVCLPNPGMMLDLQECWISGWGATYEKGTSDVLNAAMVPLIEPSKCNS
 KYIYNNLITPAMICAGFLQGSVDSCQGDSSGGLVTLKNGIWWLIGDTSWGSGC
 AKALRPGVYGNVTVFTDWIYQQMRANSENLYFQGACDYKDDDDKHHHHHHH
 H

The sequences in brackets are the remainders of the secretion signal that might still be attached to the protein. The TKNNNIALMK sequence for mutant Tmprss2 D343N is highlighted with red in the total sequence.

Result sequence:

Sample ID: 616 blue

>gi|254570889|ref|XP_002492554.1| hypothetical protein [Komagataella
 pastoris GS115]gi|238032352|emb|CAY70375.1| Hypothetical protein
 PAS_chr3_0337 [Komagataella pastoris]
 GS115]gi|328353433|emb|CCA39831.1| Cell wall protein CWP1 [Komagataella
 pastoris]

MFNLKTLISTLASIAVADQTFGVLLIRSGSPYHYSTLTNRDEKIVAGGGNKKVT
 LTDEGALKYDGGKWIGLDDDDGYAVQTDKPVGTGWSTNGGYLYFDQGLIVCTED
 YIGYVKKHGECKGDSYGMWVKVLPADDDKDDDKDDDKDDDKDYDDDDNDHG
 DGDYYCSITGTYAIKSKGSKHQYEAIKKVDAHPHVFSVGGDQGNDLIVTFQKD
 CSLVDQDNRGVYVDPNSGEVGNVDPWGELTPSVKWDIDDGYLIFNGESNFR
 SCPSGNGYSLSIKDCVGGTDIGLKVWEK

The band suspected to be Tmprss2 from Cycle 3.2 that had passed through IMAC in culture supernatant was identified to be a *P. pastoris* cell wall protein. There was no match for the TKNNNIALMK sequence for mutant Tmprss2 D343N in the supernatant sequence. This only confirmed that all Tmprss2 was in fact captured in the IMAC process and that it appears that consecutive iterations did accumulate dead cell material in the culture. The accumulation in this case is observed as the *P. pastoris* cell wall protein CWP1.

6 Discussion

6.1 **Experimentation with both Tmprss2-WT and -Mut cell lines**

Initially only the Tmprss2-Mut strain was supposed to be used for experiments instead of both Tmprss2-Mut and Tmprss2-WT cell lines. The Tmprss2 yield from Tmprss2-WT cell line was so low based on results from (12), that it was considered not to have enough potential even though it produced Tmprss2 in the desired autocatalyzed active form. The autocatalyzed form having higher activity when incubated with H1 was considered the preferable one for crystallization over the mutant D343N form of Tmprss2 with lower activity. The Tmprss2-WT variant was cultivated in Cycles 0-2 by accident. Because it repeatedly produced Tmprss2 only in the processed form and did not have the growth kinetics of a Mut^S strain it was suspected not to be Tmprss2-Mut. This was confirmed by PCR analysis from the preculture of Cycle 2. After being confirmed as the wrong cell line the next experiment named Cycle 3 was started by plating Tmprss2-Mut from stock culture sample from -80°C storage. The preculture used in Cycle 3 was then confirmed by PCR analysis to be Tmprss2-Mut. Because of this unintended experimentation with Tmprss2-WT cell line some additional time was spent wrestling with unexpected results. This extra time and the time spent on the experiments themselves set a very limited schedule for laboratory work for this bachelor's thesis. The limited amount of time available led to little possibility for repeating or streamlining already successful experiments, but experimentation with both cell lines made comparison between the two possible and enabled conducting the autocatalytic processing experiment by coincubation of autocatalyzed Tmprss2 in 1:100 ratio with mutant D343N Tmprss2 in Cycle 3.1. The Tmprss2-WT was also found to have higher potential for producing ample amounts for crystallization than was originally thought based on (12).

6.2 **Error evaluation**

High optical density of *P. pastoris* cultures required dilutions up to 1:1000 to be quantifiable by the measurement devices. Such high dilution ratios even when conducted in larger volumes of 10 mL and in series are susceptible to high measurement error. Measurement error may be magnified significantly in the repeated dilution steps. Precipitation or the lack of it could cause changes in turbidity resulting in incorrect OD values.

Strong UV absorbance of imidazole caused background issues in spectrophotometry even when respective imidazole buffers were used as blank reference. This made dialysis necessary prior to spectrophotometry and especially lower quantities of Tmprss2 would have been more accurately quantified after dialysis.

6.3 Hardware failures

In Cycle 1 the cable for connecting the methanol sensor and feed pump was found to be damaged and automatic feeding could not be established. This led to the reactor culture being transferred into 2 L flasks and cultivated in a hybrid approach. The AF sensor of the reactor experienced over sensitivity issues as individual droplets could hit the sensor and cause AF feeding in to the reactor needlessly. In methanol induction phase where the methanol itself works as an AF agent the AF sensor was manually turned off if it caused unnecessary AF feeding. Addition of the AF-agent 5% (V/V) Struktol J 673 meant addition of methanol as this AF agent was dissolved and diluted in methanol. All flasks excluding precultures which were in smaller volume and produced less foam were thus run in mixed feed with an initial charge of approximately 0.095% (V/V) methanol. The AF Struktol J673A has also been observed to increase recombinant protein expression and secretion in *P. pastoris* (26). The calibration of the methanol sensor was slightly affected by the extra methanol causing the set point in reactor to be higher than 0.5%(V/V).

The culture from Cycle 2.3 was spoiled during the centrifugation step as the centrifuge lid's magnetic lock malfunctioned, causing the culture to stay in the centrifuge and in room temperature for more than 12 h. The batch was presumed to be spoiled after such a long time and was discarded.

6.4 Semi-sterile working conditions and contamination

The cultivation methods for repeated iterations cannot be considered entirely sterile as the culture was removed from the sealed reactor. The culture was transferred into clean, but not sterile centrifuge flasks which were open for balancing prior to centrifugation. Another significant breach of sealed reactor conditions occurred in Cycle 3 where the membrane in the methanol probe was ripped during assembly and had to be replaced after the reactor had already been autoclaved.



Figure 34. Reactor contamination seen as a yellow culture adhering to the reactor wall and baffle.

During Cycle 3.3 an anomaly was detected adhered to the reactor wall. A sample was taken from the reactor for microscopy and plating. During microscopy at x1000 magnification only yeast cells could be seen in the sample. The sample was plated on YPD plates with and without ampicillin. After an overnight incubation at 30°C unknown microbial growth was detected on both plates. Because of the fast growth rate and ampicillin resistance of the contaminating organism it was presumed to be a laboratory strain of *Escherichia coli*. After the contamination was confirmed the cultivation was stopped and the reactor was directly autoclaved with its contents.

6.5 IMAC issues

In general, the Äkta start system with NiSepharose™ excel material column worked well with slow flowrates and overnight loading. Using the system was a significant improvement over the simple setup for Cycle 0 which was mostly manual. The Äkta start system featured constant measurement and real-time datalogging of UV-absorbance, flow rate, elution buffer gradient, conductivity and pressure. The greater amount of real time and recorded information as well as the automatic fractioning of the elution fractions made evaluating the process easier and diagnosing issues possible as they occurred. The best example of this can be seen in Appendix 9.2.2 Chromatogram Cycle 1.2. IMAC in Cycle 1

suffered from increased back pressure and eventually blocking of the sampling flow path due to precipitation of reactor media components in the culture supernatant. The precipitation was increased when the pH of the culture supernatant was adjusted above 6 as was recommended for using NiSepharose™ excel material by the manufacturer. This led to failure to collect the fractions from the C1.2 supernatant cultivated with iteration method B as the IMAC had to be aborted and Äkta start washed due to tubing being blocked by precipitate. During the following Cycles the pH was not adjusted as it was already in the range of 5.8-6 and considerably less precipitation occurred.

It appeared that the expression of Tmprss2 was lost or the capture of the protein was unsuccessful IMAC in Cycle 2. No Tmprss2 was detected in the elution fractions by SDS-PAGE or WB in Cycle 2.1. This is likely caused by user error where the fractionation was started too late and the peak elution fractions were pumped into waste instead of being collected. The values for UV-absorbance in Cycle 2.1 IMAC seen in Appendix 2.4 followed the same pattern as they did in the IMAC for other Cycles i.e. Cycle 3.2 Appendix 9.2.6 where Tmprss2 was detected WB. The higher initial peak when 200 mM Imidazole buffer is pumped through the column which then logarithmically levels to a plateau was a repeated pattern in all elutions, but it was the clearest in elution with 200 mM imidazole elution buffer. This pattern seems to be indicative of material first being eluted off the column and then the UV absorbance settling to the level caused by the imidazole, which corresponds well with the fact that the first fractions during the initial peak in 200 mM imidazole elution also gave the strongest signal for Tmprss2 both on WB and in photospectrometry. The same pattern was observed also when introducing 500 mM imidazole elution buffer to the column except the difference between the peak and the level UV-absorbance it settles to afterwards was considerably smaller. This is well in line with what was detected with WB and spectrophotometry where most of the Tmprss2 is seen eluted as a gradient mostly during 200 mM imidazole elution and again during 500 mM imidazole elution, but only in trace amounts. Considering the recorded UV-absorbance graph seen in Appendix 9.2.3 it is likely that Tmprss2 was produced in C2.1, but it simply wasn't successfully fractionated from IMAC due to user error. In Cycle 2.2 Tmprss2 was not detected on WB or with spectrophotometry and loss of expression was suspected once more. It is more likely that the reason was failed IMAC once again. Because of the known tendency of Tmprss2 to degrade if not stored in at least 4°C an improvement was tested on C2.2 IMAC. Instead of letting the culture supernatant stay in RT the Schott flask containing the culture supernatant being loaded onto the column overnight was placed in a bucket filled with ice. Unfortunately, as the ice melted the bottle started to float at an angle where the intake tube for sample loading was not submerged and air was pumped onto the column for several hours. This can be seen in the Appendix 9.2.4 where the UV-value is not steady but very unstable for a large portion of the loading phase while air is being pumped onto the column. The drying of the

column would have likely caused the loss of the Tmprss2 already bound during loading. When the issue was discovered the loading was directly continued by resubmerging the intake tubing and levelling the bottle. This was not enough as the dry column would not be able to bind the consequently loaded Tmprss2 without being first saturated with alcohol to remove the air and then equilibrated with equilibration buffer.

Some of the produced Tmprss2 would have likely degraded during IMAC as well. IMAC was conducted overnight in room temperature likely resulting in loss of Tmprss2. This may be a reason for the higher Tmprss2 yields per culture supernatant volume used in IMAC in Cycle 0 where IMAC was conducted in a few hours instead of an overnight process. The reason for using such a long loading process with the Äkta start system in comparison to the Cycle 0 setup was that the volume of the column was slightly smaller 5 mL instead of 6 mL while the volume of culture supernatant was more than double the volume compared to the 500 mL in Cycle 0. Because of the time-consuming preparation of the culture supernatant by centrifugation and filtration steps it was not possible to complete the preparation and IMAC during a single day. The solution to the loading phase in the IMAC process with Äkta start often taking 10h or longer was conducting it overnight.

An alternative to using NiSephrose™ excel in a column could be adding it directly in to the supernatant after filtration. The supernatant with NiSephrose™ excel could then be left for capture overnight in 4°C while being end over end mixed. The NiSephrose™ excel could be separated after the overnight incubation by centrifugation at 4°C. After separation, the NiSephrose™ excel material with bound Tmprss2 could be resuspended in 200 mM imidazole buffer for elution. Once separated again from the liquid fraction by centrifugation the excel material could be renewed by equilibration and stored in ethanol for future use. The Tmprss2 now eluted in the imidazole buffer would then be ready for dialysis and analysis by SDS-PAGE, WB and spectrophotometry. Using this method would allow performing the entire IMAC process in 4°C avoiding Tmprss2 degradation while allowing hours of contact between the culture supernatant and the resin material. This method also has no risk of disrupting the column with either air or precipitate as the NiSephrose™ excel material is floating freely in the culture supernatant. Same could be achieved by moving the entire Äkta start IMAC setup into a cold room at 4°C, but the issues with precipitation would likely only increase with this option as solubility decreases in low temperature.

6.6 Loss of protein during concentration

It would appear that some of the produced Tmprss2 was always lost in the concentration step. This was observed by comparing the individual fractions and the final concentrated total fraction as well as the total fraction prior to concentration. The clearest indication of protease loss during concentration was demonstrated in Cycle 4 where a lot of Tmprss2 from the dialyzed unconcentrated total fraction was lost during the concentration step. The initial amount of Tmprss2 in the unconcentrated total fraction for Cycle 4 was 4,78 mg in 10 mL volume. After 10:1 concentration the amount was only 0,959 mg in 1 mL volume. The Tmprss2 concentration was increased roughly 2:1 in 10:1 concentration while it appears most of the Tmprss2 was lost. This gives reason to assume that the actual amounts of Tmprss2 produced would be higher than the final values measured after concentration seen in Table 3. The loss of Tmprss2 in the concentration step could be countered by utilizing a different concentration technique that does not cause such high loss of Tmprss2.

6.7 Precipitation issues

The standard single-cell protein media recipes work with recombinant strains. However, they are not perfect or unoptimizable. The precipitation of the salts occurs during steps such as medium preparation, combining base medium and hexametaphosphate fraction in the reactor and IMAC. The salts may also precipitate in high cell densities without it being detected causing error in cell density measurement through OD because of the increased turbidity caused by precipitated salts (21). One approach to solving this issue would be reducing the concentration of salts in the basal salt medium to $\frac{1}{4}$ of the amounts given in the recipe (27). This reduction has been observed to have no negative influence on cell growth rate, biomass yield or the level of recombinant protein production (29).

6.8 Running reactor in ultra-high cell density conditions

The production of Tmprss2 could likely be improved by running the reactor in ultra-high cell density conditions in the range of OD > 500; WCW > 400 g/L which were not reached with the method aimed for mitigating the time needed for each cultivation step used in this thesis work. The ultra-high cell density conditions would be achievable by prolonging the glycerol feed phase and using additional oxygen in the air flow to keep pO₂ above 20%. The higher cell density culture would likely consume methanol at a higher rate compared to the lower cell densities used. This should result in greater amounts of produced Tmprss2 in the culture supernatant. The iterative methanol induction cultivation mode makes reaching the ultra-high cell density more efficient as the cell density is retained from the initial iteration to the following ones mitigating the effect of the additional time required for the glycerol feed phase.

6.9 Continuous cultivation with perfusion membrane for harvesting and direct IMAC with a two-line semi-continuous setup

When moving forward with cultivation techniques the semi continuous iterative cultivation mode could be developed further into a truly continuous one. The harvesting phase interrupting cultivation every 48h and causing stress as well as a lag phase in the cell growth could be avoided by utilizing a perfusion membrane. The culture supernatant could be collected through this perfusion membrane while leaving the cells in the reactor at a rate keeping the volume of the reactor constant. The reactor volume could be exchanged faster by using mixed feeding of methanol feed and the reactor media. The reactor media used in feeding could contain lower concentrations of its components compared to the reactor media initially used to fill the reactor for glycerol phase as it would have only 48h retention time in the reactor. The continuous flow from this system could be filtrated and directed onto a two-line IMAC system where one column would be in loading phase while the other one is going through elution and renewal phases. By alternating between the two columns there would be no need to pause the IMAC process regardless of the need to pump other materials on the column separately from the culture supernatant during elution and renewal steps. This continuous process would minimize Tmprss2 degradation over time by quick loading of the culture supernatant directly from the reactor to the column and remove the cell harvesting step entirely.

7 Conclusions

NiSepharose™ excel was repeatedly demonstrated to be a robust material for Tmprss2 IMAC. No issues with stripping of the immobilized nickel ions from the NiSepharose™ excel was observed at any point. In comparison, the Ni-NTA column used for Cycle 0 was completely stripped by the culture supernatant in a single IMAC run. The issue with stripping the Ni-NTA column could be countered by adding nickel directly into the supernatant in the form of nickel sulphate prior to IMAC. The NiSepharose™ excel material was capable of performing direct IMAC without the addition of nickel sulphate and presented strong binding capability proving to be the better option for IMAC. The same HisTrap™ excel 5 mL column could be used for IMAC throughout this thesis work. The column required maintenance because of incidents in which air or precipitate was pumped onto the column. The column only had to be inverted, saturated with ethanol and equilibrated in between rounds of IMAC to keep it from being blocked or getting dry. Despite the stress to the column it was still functioning exactly as intended.

The iterative methanol cultivation mode was shown to work in reactor scale in Cycle 3. The process could also be optimized by adding pure oxygen to the airflow during glycerol growth phase to shorten the feeding phase from an overnight process to approximately six hours. The additional oxygen removes dissolved oxygen limitation and allows glycerol feed to be fed to the reactor at an increased flow rate. This would also enable opting out for running the reactor in ultra-high cell density conditions higher than what was done with this method optimized for shortening the time to reach the induction phase.

While the fresh media and the reactor cultivation setup provide everything required for maintaining the culture, in theory the iterations could be repeated limitlessly without losing expression creating a semi continuous process. However, build-up of dead cell material or other material contained in the cell pellet can cause issues as well as unused media or feed components as some are always left in the remaining 1/3 reactor volume in each iteration. Building up of material can cause precipitation in the reactor and purification steps as well as increased need for filtration of supernatant for downstreaming. In cases of build-up of media components their concentration can be checked between each iteration or measured real time for example in the case of salinity. The concentrations can then be adjusted accordingly in the fresh media added between iterations. Dilution of media can also occur if the 1/3 remainder has been depleted. This could also be countered by increasing the concentrations of media components in fresh media added between iterations. The build-up of materials in the cell pellet or the cell density itself can be controlled by the amount of WCW that is returned to the reactor.

Removing the cells from the sealed reactor as well as inserting them back increases the risk of contamination. However, this risk is somewhat reduced by the high cell density of *P. pastoris* at expression phase. The risk of contamination can be further reduced by sterile working conditions and adding antibiotics into the reactor media.

Tmprss2 was produced in scale of several mg which was the target for creating material which could be further purified for crystallization experiments. Highest total yield after concentration in a single iteration was reached in Cycle 3.2 (1,92 mg) as seen in Table 3. This can be considered close enough to the target 2 mg. Even higher total yield of 4,78 mg was present in the total fraction of Cycle 4 prior to concentration. However, the quality of the Tmprss2 D343N after only the initial purification was far from homogeneous and would require more processing before reaching quality suitable for crystallization. The fast 48h production cycle in iterative methanol induction mode would allow pooling the produced Tmprss2 from several successive iterations to reach higher concentrations either prior to or after IMAC. Pooling the products of several iterations would make crystallization of the desired autocatalyzed form of Tmprss2 produced by the Tmprss2-WT cell line possible. The issue with the purity of Tmprss2 D343N produced by Tmprss2-Mut could potentially be countered with using processed Tmprss2 produced with WT-Tmprss2. Autocatalyzing the uncleaved form with lower activity to process it to the truncated form with higher activity would reduce the amount of iterations required to reach ample amounts of active Tmprss2 for crystallization. Perhaps the simplest way to increase production of Tmprss2 following this thesis work would be upscaling the reactor. The developed cultivation strategy for *P. pastoris* cell lines in 2 L scale using a bioreactor in iterative methanol induction cultivation mode should be applicable also in larger reactors. The larger reactor volume should result in a higher total yield of Tmprss2 for each iteration.

It was observed that much of the protein is lost during concentration the elution fractions. This could be optimized by using a more sophisticated concentration technique i.e. repeated IMAC, diafiltration or TCA precipitation.

8 References

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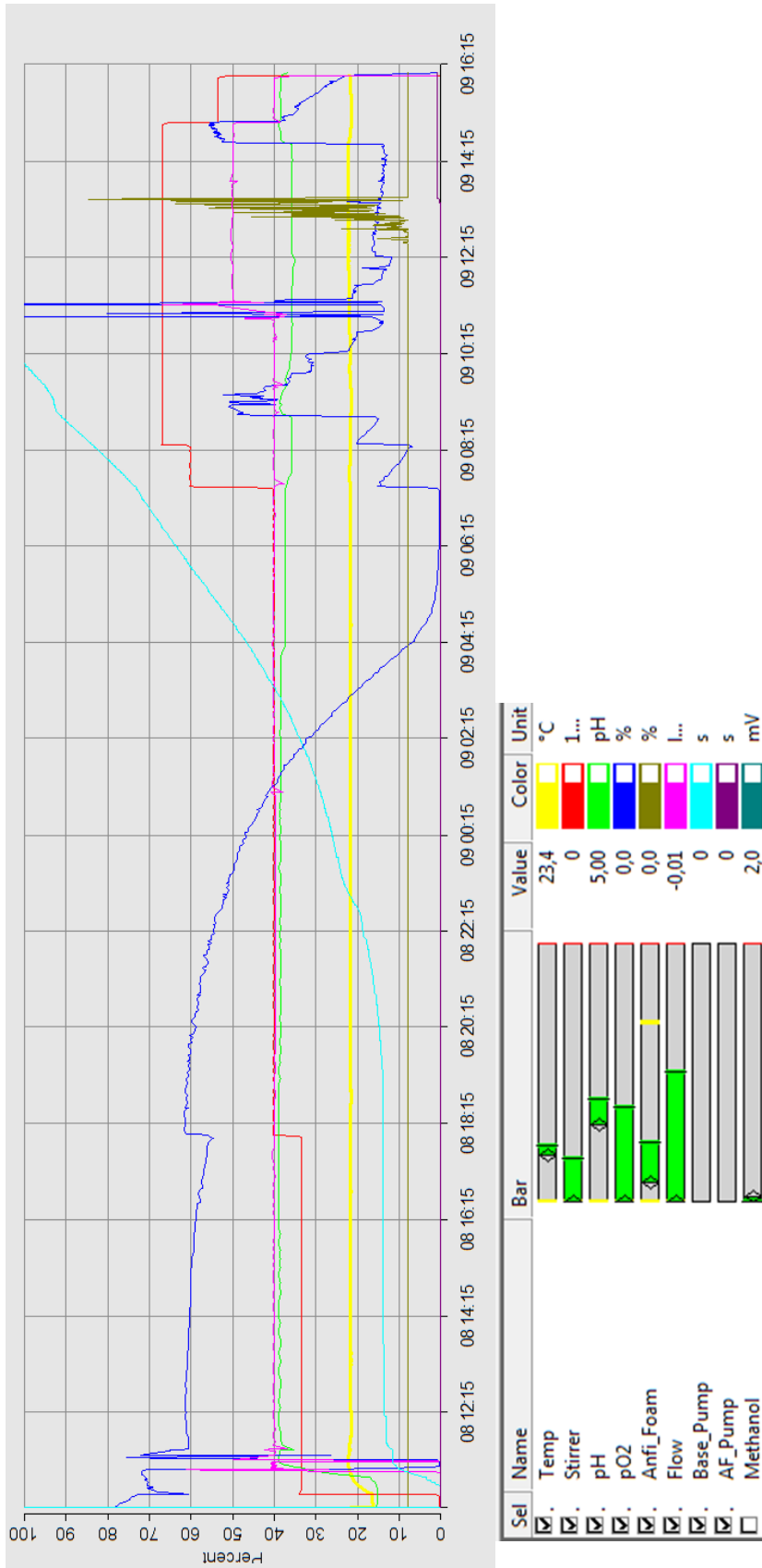
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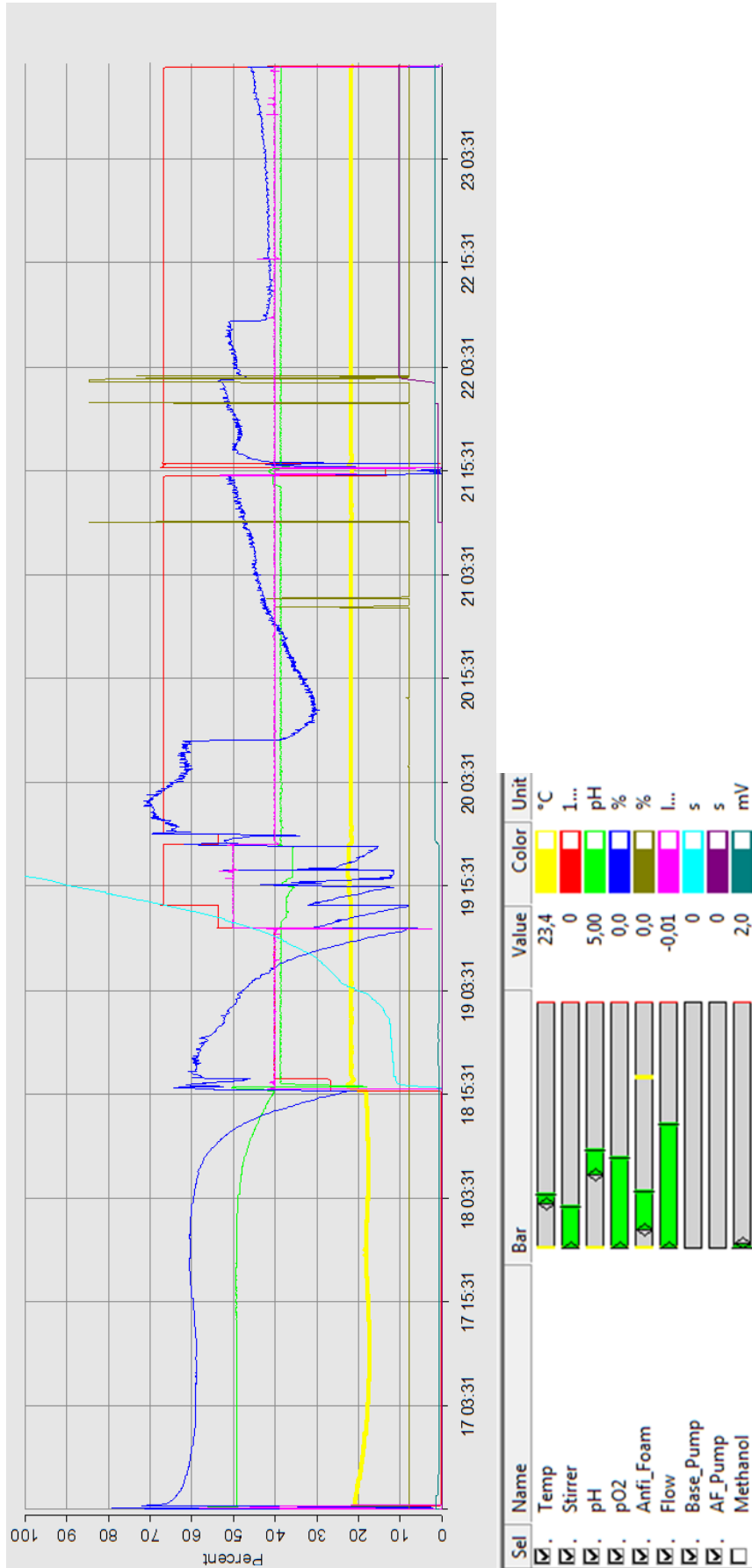
9 Appendices

9.1 *Appendix 1. Reactor logs*

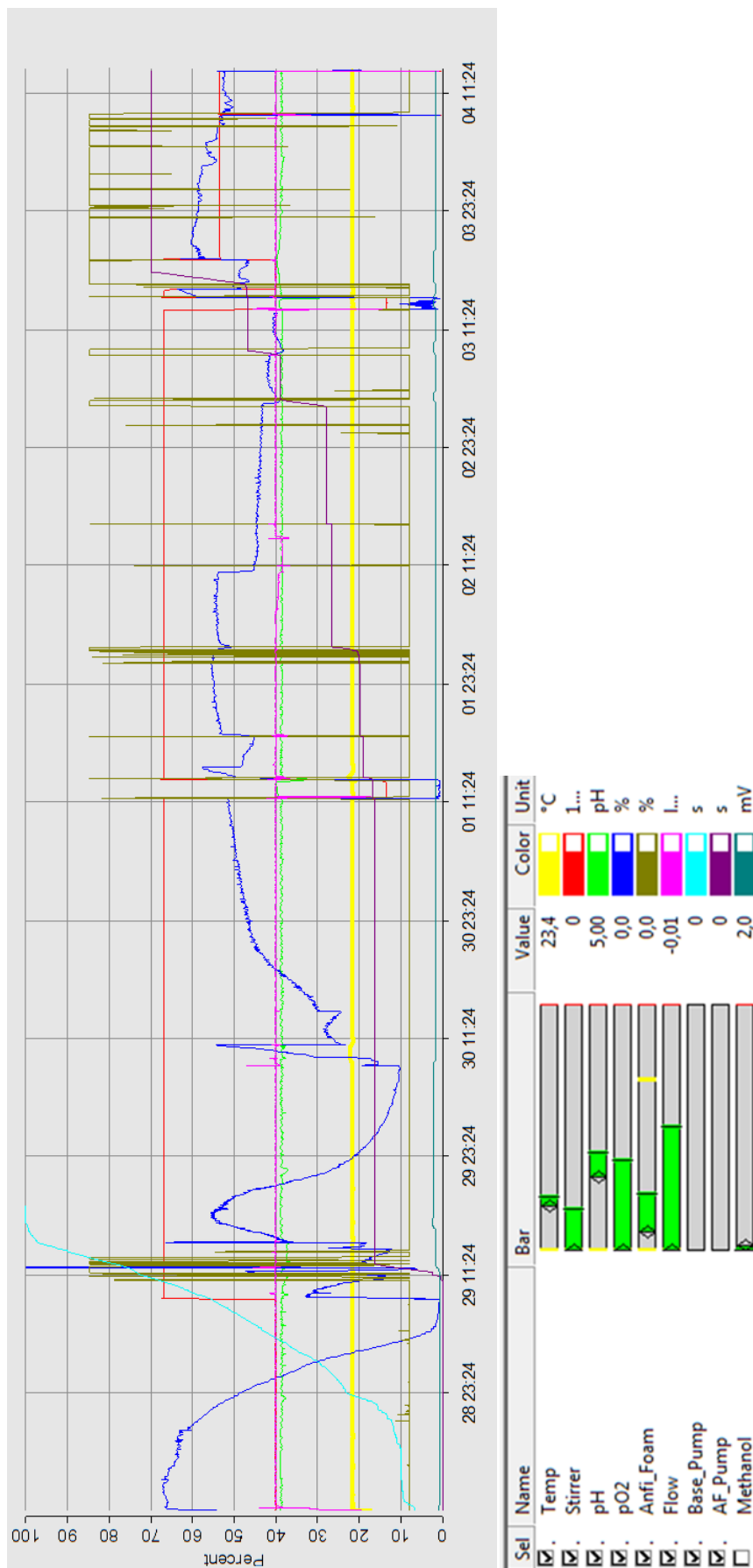
9.1.1 Reactor log Cycle 1. Tmprss2-WT glycerol growth phase in 2 L Labfors 3 reactor.



9.1.2 Reactor log Cycle 2. Tmprss2-WT in 2 L Labfors 3 reactor iterative methanol induction cultivation mode



9.1.3 Reactor log Cycle 3. Tmprss2-Mut in 2 L Labfors 3 reactor iterative methanol induction cultivation mode



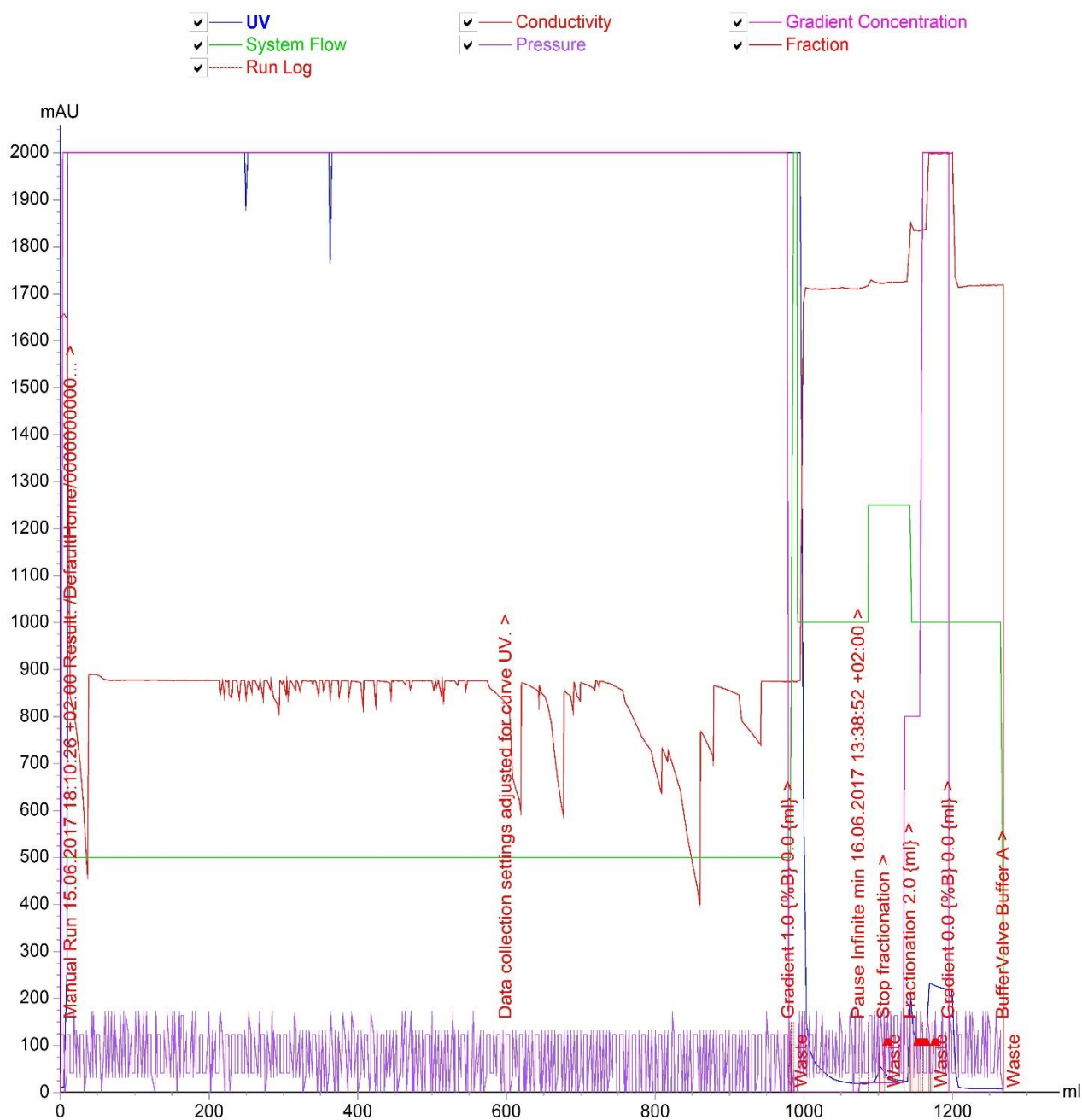
9.2 Appendix 2. Äkta start IMAC logs

UNICORN start 1.0

16.06.2017 15:53:23 +02:00

Result: WT 20170616 Run 2

9.2.1 Chromatogram Cycle 1.1



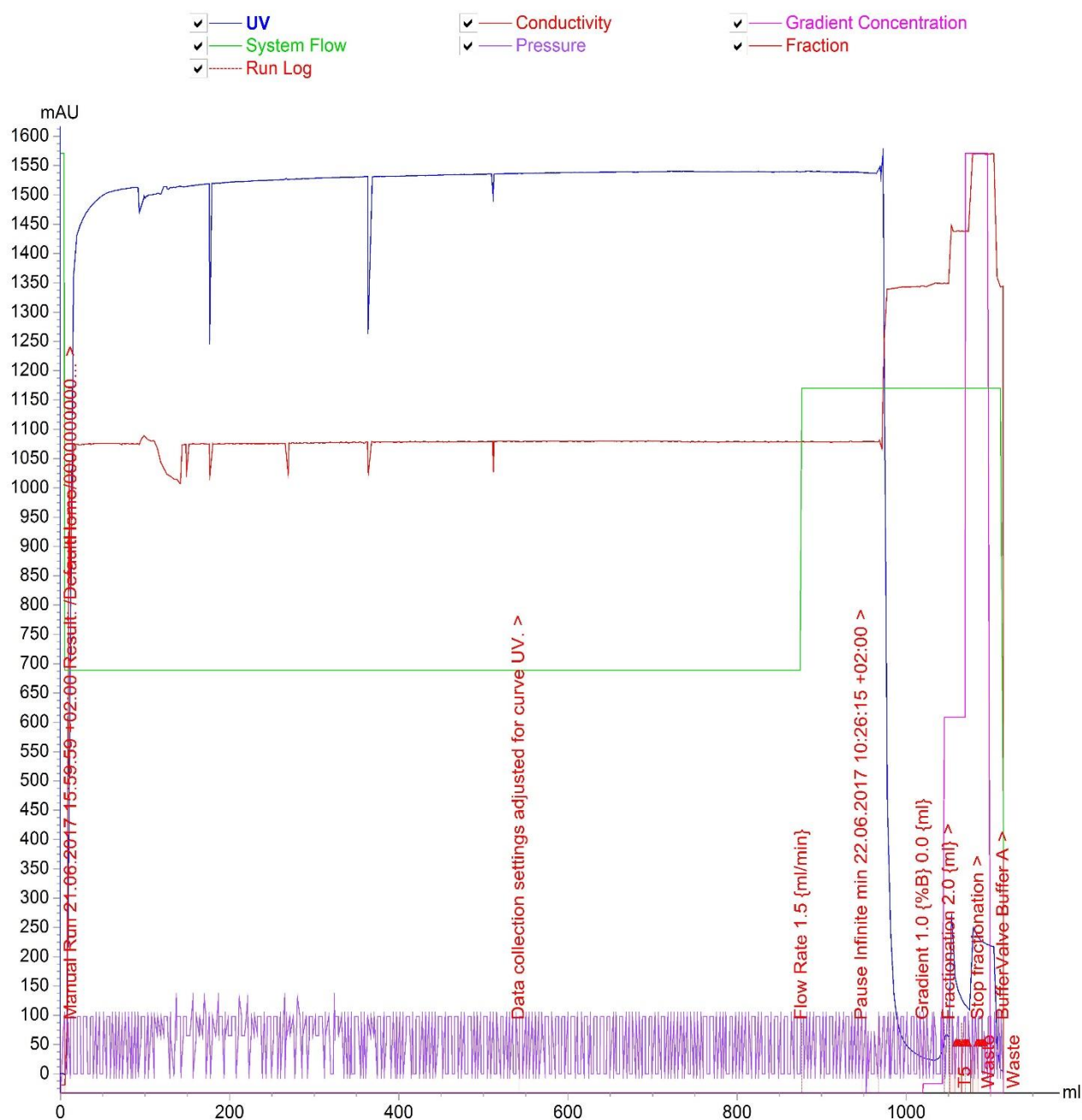
06.07.2017 13:24:00 +02:00

UNICORN start 1.0

22.06.2017 12:28:07 +02:00

Result: Tmprss2 WT 20170622 Run4

9.2.3 Chromatogram Cycle 2.1

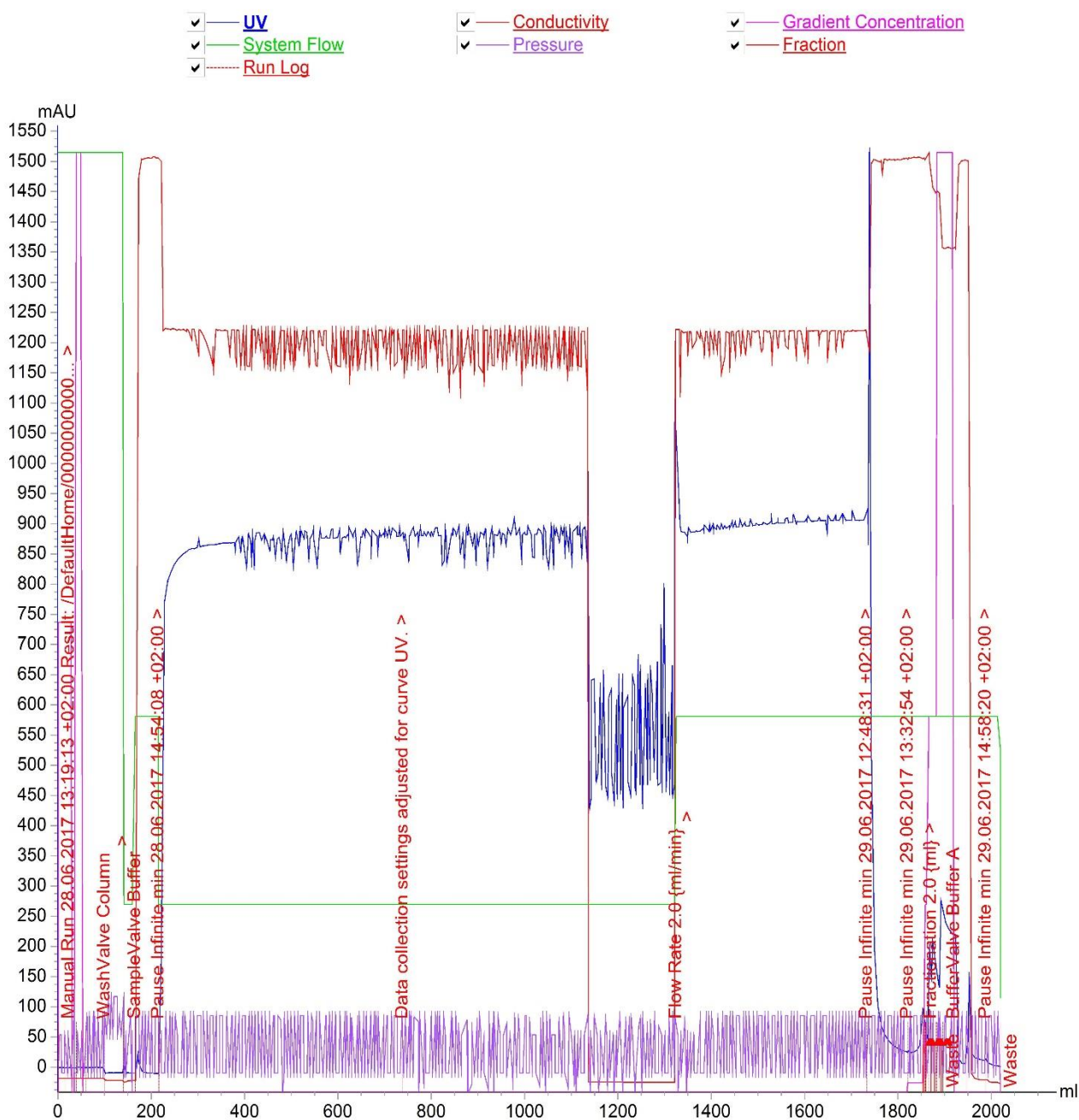


UNICORN start 1.0

28.06.2017 13:19:11 +02:00

Result: Manual Run Cycle 2.2

9.2.4 Chromatogram Cycle 2.2

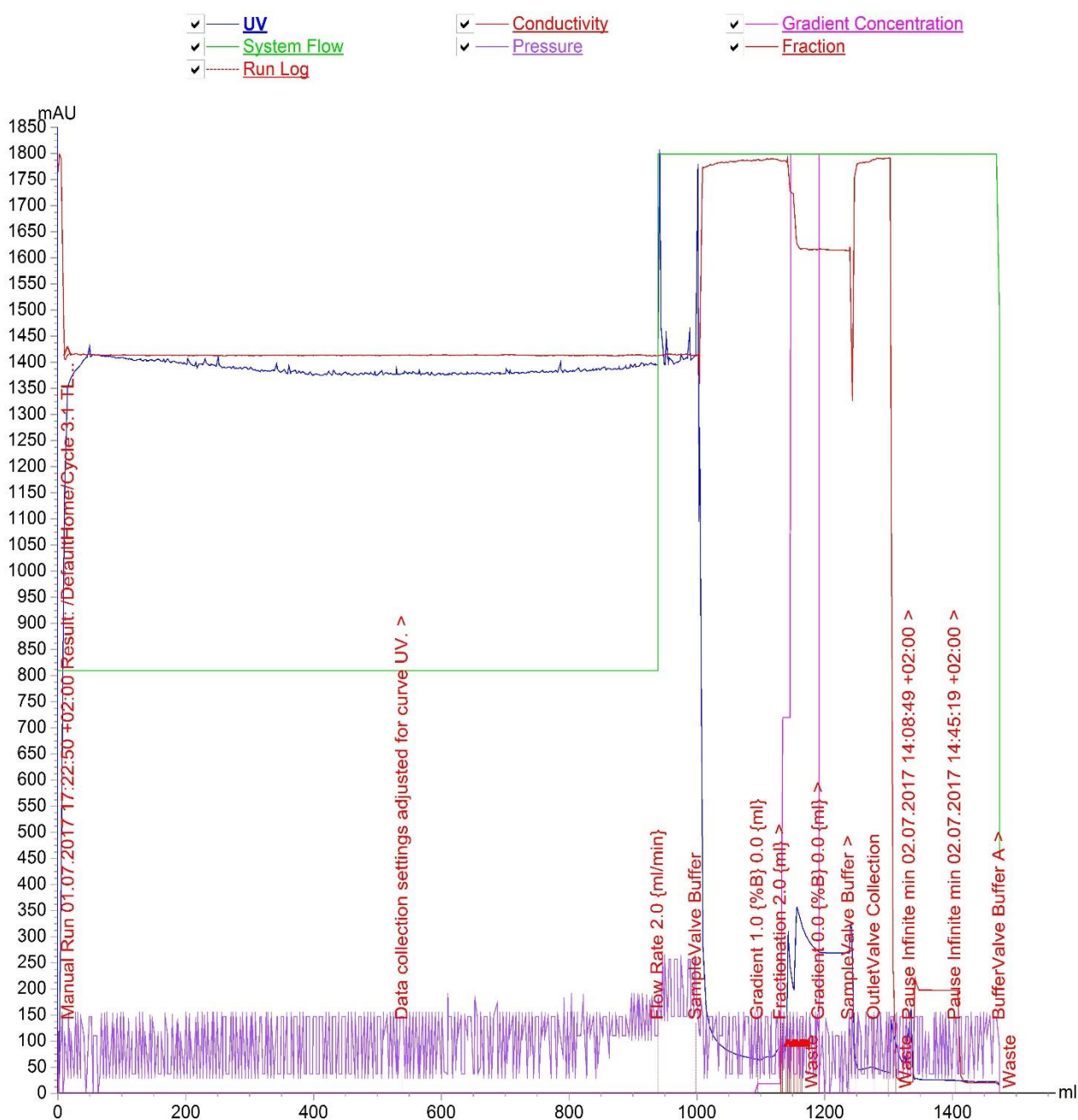


UNICORN start 1.0

01.07.2017 17:22:50 +02:00

Result: Cycle 3.1 TL17

9.2.5 Chromatogram Cycle 3.1

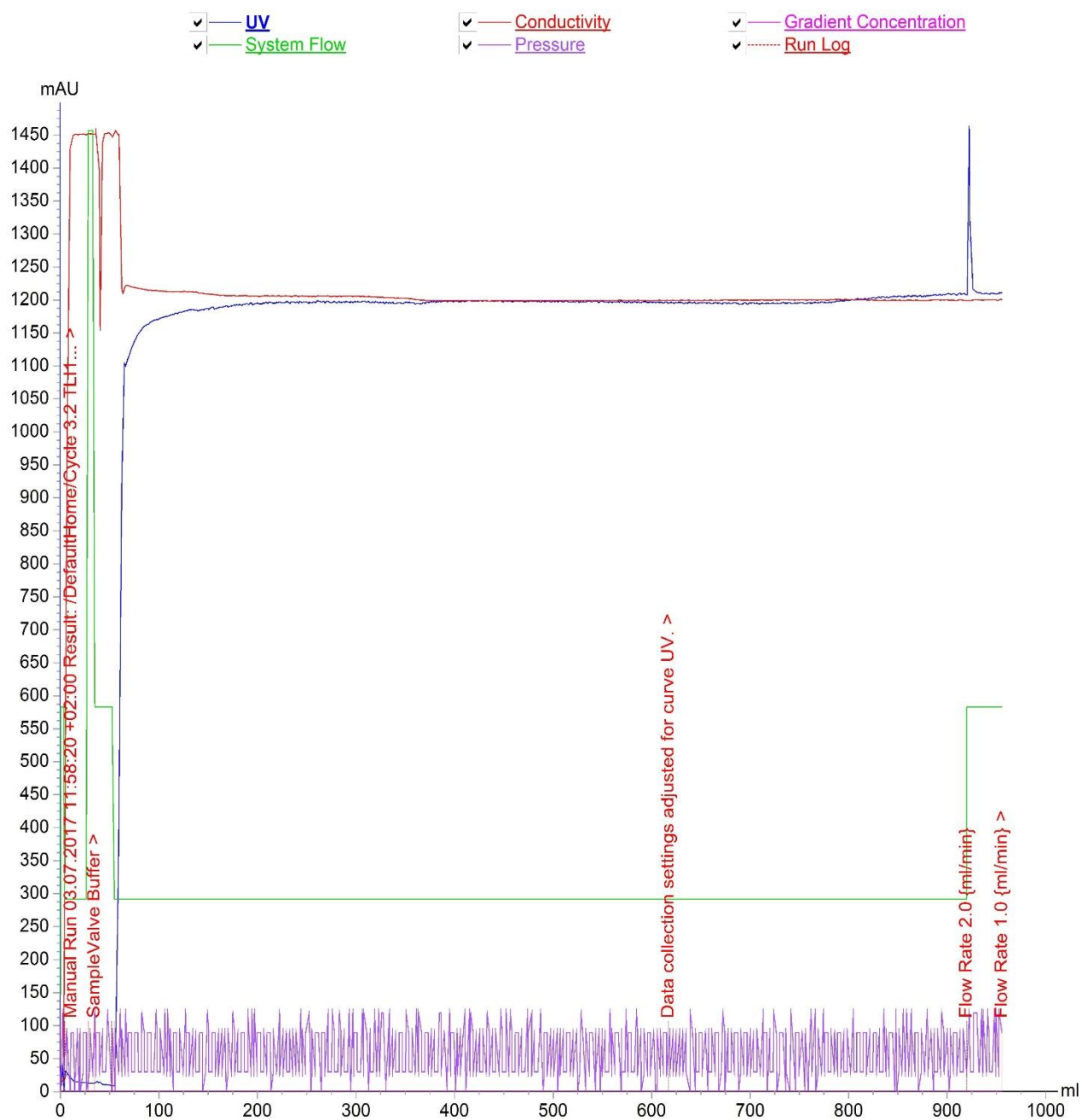


UNICORN start 1.0

03.07.2017 11:58:19 +02:00

Result: Cycle 3.2 TL17

9.2.6 Chromatogram Cycle 3.2 Loading

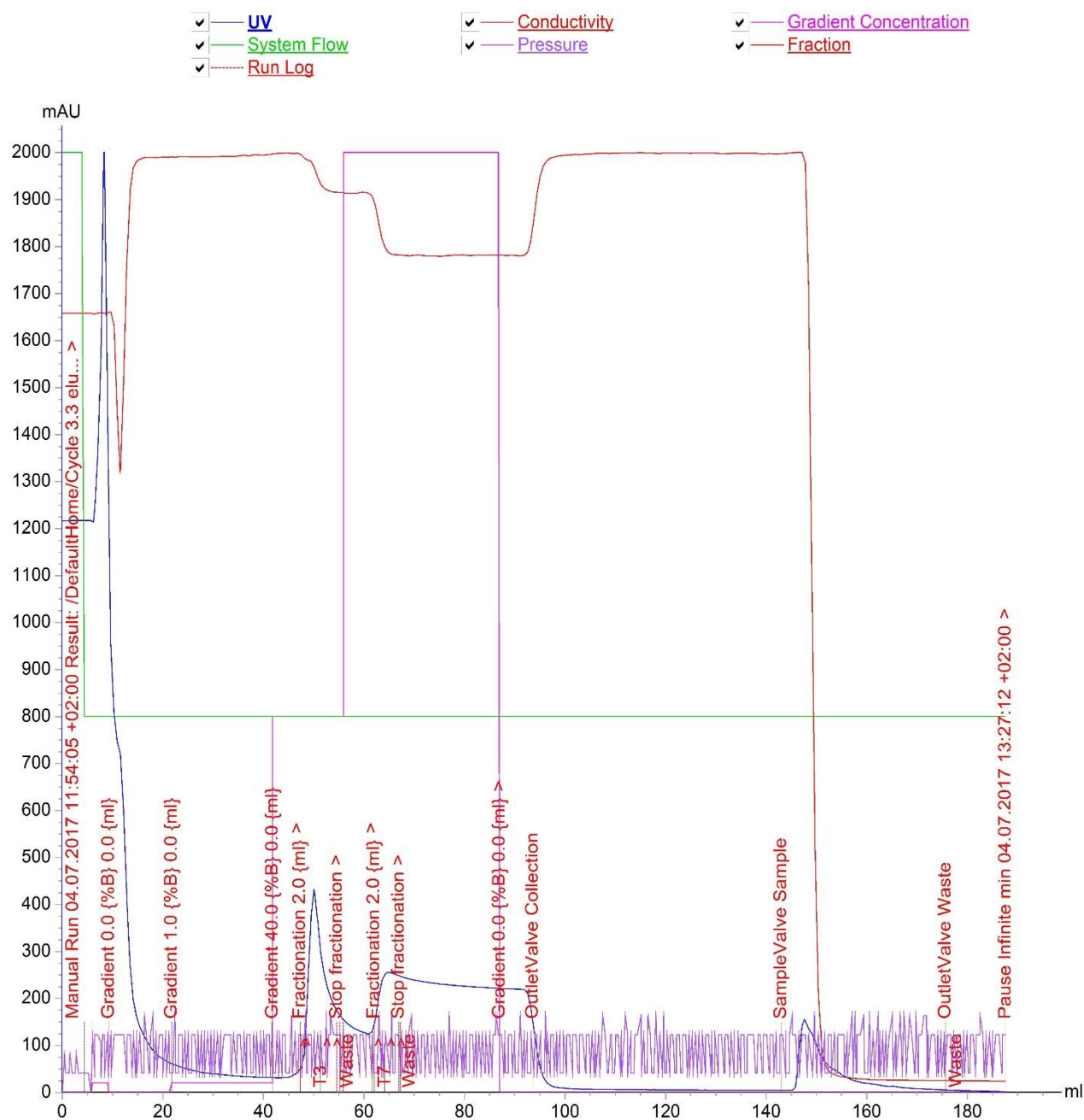


UNICORN start 1.0

04.07.2017 11:54:04 +02:00

Result: Cycle 3.3 elution TLI17

9.2.7 Chromatogram Cycle 3.2 Elution



UNICORN start 1.0

9.3 Appendix 3 Materials list

9.3.1 Laboratory equipment

Purpose	Make	Model	Checked on	Variations
Air Filters for reactor	Gelman	ACRODISC CR PTFE 0.2 um	N/A	N/A
Balance	Sartorius analytic	BP 3100 S	N/A	N/A
Beaker	VWR/Schott Duran	N/A	N/A	50ml-2L
Biological Safety Cabinet	Thermo Fisher Scientific	HerasafeKS 15	30/9/2016	N/A
Bioreactor	Infors AG	Labfors 3 2 l fermentor system	28/07/2017	N/A
Centrifuge for small scale	Thermo Fisher Scientific	Heraeus Fresco 17 centrifuge	6/2017	N/A
Centrifuge for harvest	Thermo Fisher Scientific	Sorvall RC 6+ Centrifuge	13/10/2016	N/A
Centrifuge for concentration	Thermo Fisher Scientific	MULTIFUGE 35-s	7/2014	N/A
Cold storage -20 °C	Liebherr	Öko Super	1/6/2017	N/A
Cold storage 4 °C	Liebherr	SN-ST	6/2017	N/A
Cold storage -80 °C	Sanyo	Ultra low freezer MDFU53V	N/A	N/A
Cooling circulator	Julabo	F34	6/2017	N/A
Corning® centrifuge tubes	Sigma-Aldrich Co.	Corning® centrifuge tubes	N/A	15 - 50 ml
Electrophoresis cell	Bio-Rad Laboratories	N/A	N/A	N/A
Electrophoresis power source	Bio-Rad Laboratories	PowerPacc Basic	6/2017	N/A
Erlenmeyer flask	Schott Duran	N/A	N/A	20ml-2L
Filters for vacuum filtration 0.45 um	Sartorius Stedim Biotech GmbH	Cellulose Acetate Filter	N/A	N/A
Fume hood	HESCO	Labcontrol	2016	N/A
Glockfilters for sterile filtration	Sartolab-P20	Sartolab-P21	N/A	N/A
High accuracy balance	Sartorius analytic	200 S	N/A	N/A
Incubation chamber 30°C	gbf heraeus	b20	6/2017	N/A
Incubation chamber 37°C	gbf heraeus	b20	6/2017	N/A
Inkscape software	Open Source Software	Version 0.92.2	N/A	N/A
IRIS- software	Infors	N/A	N/A	N/A
Laboratory coat	N/A	N/A	N/A	N/A

Magnetic stirrers	Heidolph	MR 3001	6/2017	100-1200 Rpm
Measuring cylinder	Vitlab	N/A	N/A	N/A
Membrane tubes for dialysis	Spectrumlabs	SpSpectra/PorTM dialysis tubes	6000-8000 MWCO	N/A
Methanol sensor & probe	Raven biotechnick	Methanol Detector and Sensor	6/2017	N/A
Millipore water system	MERCK	Advantage A10	N/A	N/A
Mixers for staining	Biometra	WT 12	6/2017	5-50 Rpm
MS Office package software	Microsoft Corporation	2016 Version	N/A	N/A
NanoDrop	Thermo Fisher Scientific	NanoDrop 2000c	6/2017	N/A
NanoDrop software	Thermo Fisher Scientific	Version 1.4.2	N/A	N/A
Nitrile glowes	Starlab International GmbH	Star guard comfort	N/A	N/A
OD meter	Biochrom Ltd	WPA CO 8000 Biowave Cell Density Meter	6/2017	N/A
pH-meter reactor	Mettler Toledo	165 DPAS SCKBS22	N/A	N/A
pH-meter	Metler Toledo Laborph	Sevenmulti S40	6/2017	N/A
Pipettes	Gilson	Pipetman	N/A	1-1000
Pipetus autopipette	Hirschmann Laborgeräte	Pipetus	N/A	N/A
Reusable bottle top filter unit	Nalgene Co.	NALGENE Reusable bottle top filter unit	N/A	N/A
Polyvinylidene difluoride (PVDF) membrane	MERCK	Immobilon-P	N/A	N/A
Premade SDS gels	Bio-Rad Laboratories	Mini-PROTEAN precast gels	N/A	N/A
Pump for Cycle 0 affinitycapture	Watson Marlow	101U	6/2017	N/A
Pump for Glycerol and Methanol feed	Watson Marlow	101U	6/2017	N/A
Pump for manual capture	Watson Marlow	101U	6/2017	N/A
Pump for sterile filtration	Ismatec	Masterflex	6/2017	N/A
Pump for vacuum filtration	KNF LAB LABPORT	N/A	6/2017	N/A
Reaction caps (EPI's)	Ependorf	N/A	N/A	1.5-2 ml
Reactor pO2 Meter	Mettler Toledo	pO2 probe	N/A	N/A

Safety goggles	N/A	N/A	N/A	N/A
Scanner	Canon	CanoScan LiDe 700F	6/2017	N/A
Schott flask	Schott Duran	N/A	N/A	50ml-2L
Self packed column	Bio-Rad Laboratories	Econo-Column® Chromatography Column, 5.0 × 30 cm	N/A	N/A
Shaken incubation chamber 28C	Infors	Multitron 2/50	N/A	N/A
UNICORN start software	GE Healthcare Bio-Sciences AB	Version 1.0	N/A	N/A
UV-meter Cycle 0	Bio-Rad Laboratories	Econo UV-Monitor	6/2017	N/A
WesternBlot device turbo	Bio-Rad Laboratories	Trans-Blot Turbo transfer system	6/2017	N/A
Vivaspintubes	GE Healthcare Bio-Sciences AB	Vivaspin	5000-10000 MWCO	N/A
Volumetric glass pipettes	Hirschmann EM techcolor	N/A	N/A	5-20 ml
Vortexer	Heidolph	Reax top	6/2017	N/A
Äkta start	GE Healthcare Bio-Sciences AB	Äkta start	6/2017	N/A

9.3.2 Chemicals and reagents

Chemical/Product name	Chemical Formula	Producer	Lot/Charge	Expiration date
All Blue Precision Plus Protein standard		Bio-Rad Laboratories	#161-0373	27/01/2017
Ammonium	NH ₄	Carl Roth GmbH + Co. KG	345232536	28/07/2019
Ammonium sulfate	(NH ₄) ₂ SO ₅	Carl Roth GmbH + Co. KG	EG2319841	N/A
Anti-MouseIgG (H+L), Ap Conjugate	Secondary anti-body	Promega Corporation	154632	28/10/2016
BCIP/NBT Color Development Substrate		Promega Corporation	242752	13/06/2019
Bromphenol blue	C ₁₉ H ₁₀ Br ₄ O ₅ S	Stock solution from HZI	N/A	N/A
Calcium Sulfate Dihydrate	CaSO ₄ ·2H ₂ O	Merck KGaA	TA285661814	N/A
cOmplete Mini, EDTA free Protease inhibitor cocktail tablets	N/A	Roche Diagnostics GmbH	14584200	7/2017
d-Biotin	Vitamin H	Sigma-Aldrich Co.	092K0703	N/A

Dipotassium phosphate	K ₂ HPO ₄	Carl Roth GmbH + Co. KG	493208006	27/11/2017
Disodium phosphate	Na ₂ HPO ₄	AppliChem GmbH -An ITW Company	51015167	9/2020
Dithiothreitol	C ₄ H ₁₀ O ₂ S ₂	Stock solution from HZI	N/A	N/A
Ethanol	C ₂ H ₆ O	J.T Bakerr B.V.	1614105017	01/06/2021
Glycerol 100%	(HOCH ₂) ₂ CHOH	Merck KGaA	K48734892706	N/A
Glycine	C ₂ H ₅ NO ₂	Carl Roth GmbH + Co. KG	246244253	11/05/2020
HisTrap™ excel 5ml	N/A	GE Healthcare Bio-Sciences AB	10248029	5/2018
hydrogen chloride	HCL	Carl Roth GmbH + Co. KG	5042229	26/11/2018
Imidazole	C ₃ H ₄ N ₂	AppliChem GmbH -An ITW Company	3K007109	N/A
Instant Blue	Coomassie staining solution for protein gels	Expedeon Ltd.	161013100	10/8/2018
Magnesium chloride	MgCl ₂	Carl Roth GmbH + Co. KG	329108174	N/A
Magnesium sulfate heptahydrate	MgSO ₄ ·7H ₂ O	J.T Bakerr B.V.	F46162	N/A
Methanol 100%	CH ₃ OH	J.T Bakerr B.V.	1421906005	10/9/2019
Monopotassium phosphate	KH ₂ PO ₄	Carl Roth GmbH + Co. KG	55223744	22/12/2018
Monopotassium phosphate	KH ₂ PO ₄	Carl Roth GmbH + Co. KG	246244041	11/05/2020
Mouse α-Flag	Primary anti-body	Sigma-Aldrich Co.	N/A	26/11/2019
Ni-NTA His-Bind	N/A	Novagen	N/A	N/A
NiSepharose™ excel	N/A	GE Healthcare Bio-Sciences AB	10247257	5/2019
Nonfat dried milk powder	N/A	AppliChem GmbH -An ITW Company	4S008807	N/A
Peptone	N/A	BD Biosciences Advanced Bioprocessing	1033562	11/02/15
Potassium chloride	KCL	Carl Roth GmbH + Co. KG	446250961	10/2020
Potassium hydroxide	KOH	Carl Roth GmbH + Co. KG	488101723	N/A
Potassium sulfate	K ₂ SO ₅	Merck KGaA	AM0971053703	N/A
PTM1 trace salts	Stock solution from HZI	N/A	from 05022015	N/A

Sodium azide	NaN ₃	Stock solution from HZI	N/A	N/A
Sodium chloride	NaCl	Carl Roth GmbH + Co. KG	486252534	28/11/2020
Sodium dodecyl sulfate	NaC ₁₂ H ₂₅ SO ₄	N/A	N/A	N/A
Sodium dodecyl sulfate	NaC ₁₂ H ₂₅ SO ₄	Stock solution from HZI	N/A	N/A
Sodium hydroxide	NaOH	Sigma-Aldrich Co.	SZBE3500V	N/A
Sodium Hexameta Phosphate	(NaPO ₃) ₆	Sigma-Aldrich Co.	BCBN0343V	N/A
Struktol J 673	AF-agent	Richard Sichler Chemikalien GmbH	N/A	N/A
Tris hydrochloride	NH ₂ C(CH ₂ OH) ₃ HCl	Sigma-Aldrich Co.	SLBN8819V	N/A
Tris hydrochloride	NH ₂ C(CH ₂ OH) ₃ HCl	Stock solution from HZI	N/A	N/A
Tri-Sodium phosphate	Na ₃ PO ₄	Merc KGaA	K44157272321	N/A
TWEEN 20 (Polyoxyethylene (20) sorbitan monolaurate)	C ₅₈ H ₁₁₄ O ₂₆	Bio-Rad Laboratories	117851C	N/A
Yeast extract	N/A	BD Biosciences Advanced Bioprocessing	4339796	31/10/2019
Yeast Nitrogen Base Without Amino Acids (YNB)	N/A	Invitrogen	160114022101	N/A