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A novel bi-directional promoter system for tunable recombinant protein expression in *Pichia pastoris*

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Abstract

Pichia pastoris has become an important recombinant production host in research and industry. Recombinant protein production in *P. pastoris* is commonly regulated by the methanol inducible P_{AOX1} and constitutive P_{GAP} promoter systems. However, both are attributed to various disadvantages rendering limited degree of freedom for bioprocess engineers to tune productivity.

A promising and innovative approach to tune and control productivity is the combination of two different promoters with varying characteristics forming a bi-directional promoter system.

In this study, we successfully characterized a recombinant *P. pastoris* strain carrying both a de-repression promoter (P_{DC}) and an inducible promoter (P_{AOX1}). We cloned the model enzyme *cellobiohydrolase II* (CBHII) downstream each promoter variant and analyzed how different feeding regimes in the bioreactor activated these promoters and thus productivity. In fact, we were able to tune recombinant protein production using different feeding strategies. We determined optimal operating windows for both promoters to work individually but also concomitantly.

Summarizing, novel bi-directional promoter systems allow tunable recombinant protein production in *P. pastoris* and thus a high degree of freedom for bioprocess development and optimization.

Zusammenfassung

Pichia pastoris wurde in den letzten Jahren ein wichtiger Wirt für rekombinante Proteinproduktion in Forschung und Industrie. Rekombinante Proteinproduktion in *P. pastoris* wird üblicherweise durch das Methanol induzierbare P_{AOX1} oder das konstitutive P_{GAP} Promotersystem reguliert. Jedoch werden beiden Promotersystemen eingie Nachteile zugeschrieben, welche Biotechnologen in ihren Möglichkeiten einschränken, bezogen auf die Kontrolle und Regulation der Produktivität.

Ein vielversprechender und innovativer Ansatz um eben jenes zu ermöglichen, ist die Kombination von zwei verschiedenen Promotersystemen mit unterschiedlichen Eigenschaften, um ein bi-direktionelles Promotersystem zu erzeugen.

In dieser Studie konnten wir erfolgreich einen rekombinanten *P. pastoris* Stamm charakterisieren, welcher gleichzeitig einen de-repressions Promoter (P_{DC}) und einen induzierbaren Promoter (P_{AOX1}) trägt. Das Modell-Enzym *cellobiohydrolase II* (CBHII) wurde downstream jeder Promotervariante einkloniert und wir analysierten die Aktivierung dieser Promotoren durch unterschiedliche Fütterungsstrategien . Außerdem bestimmten wir den optimalen Bereich, indem beide Promotoren entweder einzeln oder gleichzeitig arbeiten.

Zusammenfassend, neuartige bi-direktionale Promotersysteme erlauben eine regulierbare rekombinante Proteinproduktion und darum ermöglichen sie einen hohen Freiheitsgrad für Bioprozessentwicklung und Optimierung.

Danksagung

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Introduction

Recombinant Protein Production in Yeast

Thousands of years ago, humans already used microorganisms to produce beverages and foods like beer, wine and bread, even though they could not make sense of the background behind the mechanisms yet [1-3]. It was not until the 19th century, when Louis Pasteur made a great breakthrough regarding the role of yeasts in fermentations. He demonstrated the conversion of sugar to ethanol and CO_2, which was the foundation for today's bioprocess engineering [4, 5].

Besides food industry, yeasts have become an important expression host for therapeutic and diagnostic sectors due to their capability to be easily genetically modified [6]. Especially in the medical sector, heterologous protein production plays a key role since the market value of biopharmaceuticals is considered a multi-billion-dollar business [6, 7].
Solely in the US, more than 80% of all recombinant produced proteins are used for medical purposes. The residual 20% are disseminated mainly to the industrial as well as agriculture market and some other small sectors [8].

In general, a wide range of biopharmaceuticals are produced worldwide. However, the market is dominated by only some of them, namely interferon-alpha, erythropoietin, hepatitis-B vaccine, granulocyte colony stimulating factor, insulin, human growth hormone, plasminogen activator, monoclonal antibodies and antibody fragments [7, 8].

The unicellular eukaryotic yeast *Saccharomyces cerevisiae* counts to the best studied microorganisms in biotechnology. Since *S. cerevisiae* has been established for a long period of time in fermentation processes and the food industry, it has obtained the classifications as GRAS (generally regarded as safe) by the FDA [9, 10]. Furthermore, it was the first eukaryotic organism from whom the whole genome was completely sequenced [11]. Additionally to this valuable knowledge, the short generation time, easy accessibility, cultivation and manipulation makes yeasts attractive organisms for the biotechnical industry [12].

Another major benefit of *S. cerevisiae* or yeasts in general, is the capability of performing some posttranslational modifications (PTMs), nonetheless they show a strong and fast generation time [13-16].

In general, two forms of glycosylation can be investigated on the surface of microbial produced proteins, N- or O-linked glycosylations. N-linked glycosylation appears mainly in lower eukaryotes such as yeasts, while O-linked glycosylation can be found in lower as well as in higher organisms [17, 18].

Seeing that *S. cerevisiae* hypermannosylates the N-linked sugar chains on the protein surface, it is not ideal for medical usage [19-21]. The human immune system perceives them as alien antigens and initiates an immune response. Hence, there is a great demand for host organisms with more human-like glycosylation patterns [6, 19-22]. A promising option is the methylotrophic yeast *Pichia pastoris*. Even though it also produces proteins of the high-mannose type, *Pichia* adds less mannoses, approximately 8-14 mannoses per sugar chain compared to more than 100 mannoses per chain in *S. cerevisiae* [22, 23]. This feature represents a major advantage for glycoprotein production in *P. pastoris*.

Pichia pastoris – a powerful expression host

P. pastoris belongs to the eukaryotic single celled species of yeasts, which is able to use methanol as sole carbon source [24]. Besides approximately 30 other methylothrophic yeasts, it uses the same methanol utilization pathway (MUT) to deal with this hazardous substrate. Hence, a special set of enzymes is needed to metabolize methanol and obtain energy through the degradation [25-28].

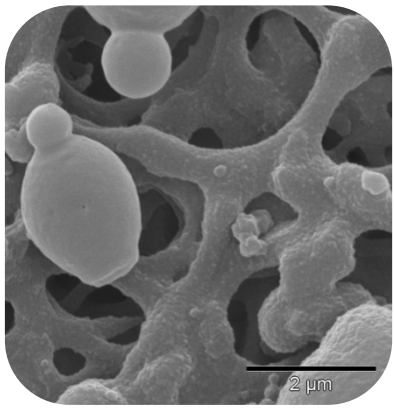

Figure 1: Electromicroscopic pitcure of a single P. pastris cell provided by Bisy e.U. (Hofstaetten/Raab; Austria)

The peroxisomal oxidation of methanol counts as the first and therefore as the most important step of the methanol metabolic pathway. The enzyme *alcohol oxidase* (AOX) catalyzes the respiratory conversion of methanol to formaldehyde and hydrogen peroxide [28]. *P. pastoris* carries two genes encoding for AOX which only differ in flanking sequences, the AOX1 and the AOX2 gene [27]. Studies addressing both alcohol oxidase genes in *P. pastoris* by Cregg JM. et al. revealed that AOX1, which is tightly regulated by methanol, contributes the most to the production of *alcohol oxidase* enzyme [28]. Further researches determined the soluble protein concentration of *alcohol oxidase* regulated by the AOX1 gene in methanol grown cells to be up to 30% [29]. Only 15% of the overall produced oxidase enzyme is contributed from the much weaker expressed AOX2 gene [28].

A way to make use of *Pichia*'s tightly regulated expression system, is replacing the AOX1 gene with a gene of interest. The heterologous sequence will then be translated instead of the native AOX1 gene [30, 31]. Due to the reason that the organism contains a second alcohol oxidase expressing gene, the cells are still capable to metabolize methanol even though they show a significantly weaker growth rate. This leads to the formation of two phenotypes: one of them is the wild type carrying both functional AOX genes (Mut^+) and the other is the Mut^S-type which is slowly growing in consequence of a dysfunctional AOX1 gene [32-34].

P. pastoris represents an interesting and powerful expression host for recombinant protein production in connection with the simple genetic modification techniques already successfully used in *S. cerevisiae* [25, 33].

Due to its capability to grow to high cell densities, it is therefore a promising model organism for researches, but also for the biotechnical industry [24]. Besides the generally attractive features of yeasts, there are a few attributes which make *P. pastoris* superior in some aspects.

Since *P. pastoris* uses a special secretory pathway for extracellular protein production and the fact that it secretes almost no native proteins into the cultivation broth, *Pichia* is a great alternative to prokaryotic expression systems [35]. Although protein purification represents an elaborate and sometimes cost intensive task, impurities should be reduced as far as possible [36]. Therefore, the secretion pathway of *P. pastoris,* which can be seen as a first purification step, simplifies subsequent downstream processes [37]. The extracellular protein production requires a secretion signal sequence to release recombinant proteins into the cultivation broth. Different secretion signal sequences have already been tested, however the most successful sequence origins from *S. cerevisiae* and is called α-factor [37, 38].

P. pastoris has been described as a high level recombinant gene expression host, which is able to express heterologous polypeptides from milligram to even gram [30, 32, 39].
Besides the capability to easily scale up *P. pastoris* cultivation processes, the regulation of recombinant protein production using methanol can be seen as a major asset for industrial fermentations [40]. The beneficial regulation by methanol relies on the special and unique promoter system of the MUT pathway in methylotrophic yeasts.

Gene regulation in *P. pastoris*

In recent years, abundant investigations were done to maximize and optimize protein expression in *P. pastoris*. A promising approach is the usage of strong and tight regulated promoters to tune productivity [41, 42].

Promoters in general are DNA regions which enable the binding of RNA polymerases needed for transcription. They are positioned upstream particular genes which encode for certain polypeptides. After the polymerase binds to the DNA, it will start to synthesize messenger RNA (mRNA) which will then be translated into proteins [43, 44]. Therefore, promoters are crucial in terms of protein expression and its regulation.

The efficiency of protein expression and as a consequence the amount of produced protein is reliant on promoter strength, which is specified by its nucleotide sequences [45].Therefore, the transcription power has a main influence on product yields. Although the amount of product is protein specific, strong promoters normally result in high product yields, while weak promoters produce less [46-48].

Since some promoters are strongly influenced by the presence of a certain carbon source because of catabolite repression and also due to the reason that this Master Thesis mainly deals with the repressive/derepressive behavior of specific promoter options, in the following paragraph the catabolite repression will be discussed shortly to explain the mechanisms behind it [49, 50].

At repressive concentrations of a particular carbon source, a dephosphorylated DNA-binding repressor complex interacts with the DNA, resulting in changes of chromatin structure. This prevents the binding of transcriptional activating factors and thereby the expression of the gene(s) [51, 52]. At low amounts of catabolite repressor, the bound repressor complex is phosphorylated by a protein kinase which results in the phenomenon of depression, meaning that the promoter region is accessible for transcriptional factors again, leading to transcription of the gene(s) [53, 54]. In fact, derepression can be used as a possible method to control promoters and as a result recombinant protein production.

For some promoters the absence of the repressive carbon source is not enough for activation. They additionally require an inducting agent to enable protein expression [50]. This as well

applies to the established and commonly used P_{AOX}-promoter of *P. pastoris,* where the presence of glucose or glycerol acts repressing at certain concentrations and is used for biomass growth [50]. On the contrary, methanol induces the activation of this promoter option, allowing protein expression [55]. Another type is the constitutive promoter which is permanently active, although the strength depends on the available carbon sources. In *P. pastoris the* constitutive P_{GAP} promoter codes for the *glyceraldehyde 3-phosphate dehydrogenase* which is strongly active on glucose and glycerol [48].
Both types of promoters have their respective advantages and are already successfully used for recombinant protein production [24].

AOX Promoter

As already previously mentioned, the enzyme *alcohol oxidase* (AOX) is regulated by two different genes. Since it introduces methanol into the MUT pathway, it represents the key catalyst in the degradation of the hazardous substrate [28]. Considering the great strength and the inducibility of P_{AOX1}, it is preferably used for heterologous protein expression [28, 41, 55]. Some parameters such as the specific growth rate of the cells [56-58], the higher efficiency of methanol utilization [56] as well as the used feeding strategy to induce the *alcohol oxidase* promoters [59, 60] have a major impact on the tunability of P_{AOX} single promoter systems and therefore on the productivity. In addition, Vogl et al. showed that by modifying transcriptional regulatory sequences of P_{AOX1}, protein expression levels could be also enhanced [61]. The assets and downsides of the *alcohol oxidase 1* promoter are summarized in Table 1.

Table 1: Advantages and disadvantages of P_{AOX1} [62]

Advantages	Disadvantages
Tight regulation/controllable	More complex process – 2 steps cultivation consisting of cell growth phase and protein expression
Repression/derepression behavior	Problem of methanol monitoring during a bioprocess
Strong recombinant protein expression, even during toxic protein concentrations for the cells [62]	Dependence of hazardous and highly flammable methanol
Uncoupling of biomass growth and protein production	

The P_{AOX1} promoter counts to the best investigated promoter options in *P. pastoris*. For its inducibility and its tight regulation, this strong promoter option is commonly used in bioengineering. However, the need of methanol presents a major weakness.

GAP Promoter

P_{GAP} is a strong and constitutive promoter in *P. pastoris*, meaning that the transcription of the *glyceraldehyde 3-phosphate dehydrogenase* gene (GAP) takes place constantly during the availability of a suitable carbon source. However, the used carbon source for cultivation can influence the strength and the constitutive behavior [48, 50]. Studies proved that using specific substrates allows to tune protein expression regulated by P_{GAP} [50, 63].

In case of oxygen deficiency (hypoxic conditions) studies showed that the expression level regulated by the P_{GAP} promoter was able to be increased [41], however heterologous protein production will be negatively influenced when toxic concentrations of the expressed target protein are reached [59].

The following Table 2 shows the contrast between the advantages and disadvantages of P_{GAP}.

Table 2: Advantages and disadvantages of P_{GAP} [48], [41]

Advantages	Disadvantages
One of the strongest promoters in *P. pastoris*	Cannot be used for toxic heterologous recombinant proteins
1 step cultivation – less complex process	Hypoxic condition led in fact to higher productivity but also to ethanol production
Similar expression levels as P_{AOX1} [48]	No tight regulation possible/uncontrolled
Nowadays promoter of choice for avoiding methanol as C-source	

The fact that no induction is needed simplifies the bioprocess, though a tight and controlled protein expression is missing.

Apart from the positive characteristics, P_{GAP} and P_{AOX1} harbor some disadvantages which could be circumvented by using other promoter options. Strong transcription initiated by P_{AOX1} and P_{GAP} may not be preferable in heterologous protein synthesis regarding for example correct protein folding and performing PTMs [41]. Therefore, weaker promoters might be more desirable in some cases. Also the avoidance of methanol as inducing agent would be of preference to look for other promoter options or even new expression systems for heterologous protein production in *P. pastoris*. Besides this commonly used promoter option, some alternatives exist which may be used for recombinant protein production. However, they are not studied as well as P_{AOX1} and P_{GAP} [41].

As shown in Table 3, a comparable strong promoter alternative to P_{AOX1} is the *formaldehyde dehydrogenase* promoter, which is also an important enzyme in the MUT pathway [64]. A main characteristic of P_{FLD1} is the ability to be inducible with methanol as well as methylamine [64]. Another strong promoter option of the MUT pathway showing high expression levels, is the *dihydroxyacetone synthase* promoter P_{DAS} [41]. Since it is an inducible promoter, it has the ability to substitute the alcohol oxidase promoter for heterologous protein production. However, due to the fact that P_{DAS} is also induced by methanol, it has the same negative effect as $P_{AOX1.}$

Although all available single promoter options in *P. pastoris* show benefits, the limitation in flexibility and in tunability represents a strong need for developing novel and innovative expression systems. A promising approach is the combination of promoters forming a bi-directional promoter system to tune and control productivity.

Table 3: Some genes of P. pastoris promoters which are used for recombinant protein expression; Table represents a short excerpt of the publication of Vogl T. and Glieder A. [41]

Gene Name	Gene Product	Regulation	Expression Level	References
AOX1	Alcohol oxidase 1	Methanol inducible	Strong	[33], [27], [30], [65]
GAP	Glyceraldehyde 3-phosphate dehydrogenase	Constitutive	Strong (comparable to P_{AOX1})	[48]
AOX2	Alcohol oxidase 2	Methanol inducible	Approx. 5-10% of P_{AOX1}	[28], [66]
DAS	Dihydroxyacetone synthase	Methanol inducible	Strong (comparable to P_{AOX1})	[27], [30], [65]
FLD1	Formaldehyde dehydrogenase	Methanol and methylamine inducible	Strong (comparable to P_{AOX1})	[64]
TEF1	Translation elongation factor 1 alpha	Constitutive and strong growth associated	Strong (comparable to P_{GAP})	[67], [68], [69]

Bi-directional Promoter Systems

Bi-directional promoter systems are specific double-stranded transcriptional elements of the DNA, which enable a double-sided (forward and reverse) protein expression. The unique orientation of promoters allows the co-expression of two (different) genes by cloning them downstream a particular promoter (see Figure 2). By combining single promoter options to form bi-directional promoter systems, the varying regulation strategies, promoter strengths and individual advantages can be used to tune and improve the cultivation and production process of a fermentation [70-72].

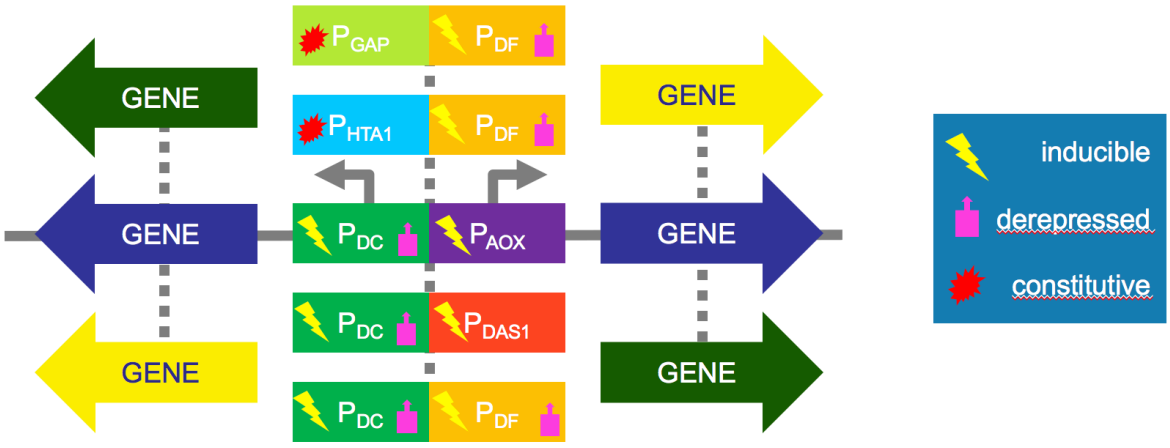

Figure 2: Schematic illustration of bi-directional promoter system combinations; Gene products of particular promoters: GAP = glyceraldehyde 3-phosphate dehydrogenase, DF = modified formaldehyde dehydrogenase, HTA1 = Histone H2A, DC = modified catalase, DAS = dihydroxyacetone synthase

The possibility to specifically activate the promoter combinations provides a greater degree of freedom. In contrast to single promoter systems, bi-directional promoter systems are able to carry two different promoter options downstream each promoter. This means that two genes can be transcribed individually or simultaneously, which is a major advantage of such systems. For instance, in case of producing a hard folding protein it could be beneficial to express a chaperone (helper protein) prior to the target protein. This can be achieved by producing the chaperone under the control of a constitutive promoter while the target gene will be regulated by an inducible promoter. Therefore, gene co-expression of such factors will significantly increase and enhance productivity as well as protein activity [73]. Besides that, bi-directional expression would open up new possibilities such as dimeric protein production or expression of enzyme together with a redox partner. By using two different genes

downstream each promoter option, it is possible to directly control concomitant or individual expression of certain genes [71, 72].

Currently, state of the art used techniques for co-expression of two genes in *P. pastoris* are based on the usage of two individual expression vectors, each carrying one of the desired genes. The vectors can be transformed in the same strain at once or first transformed in two different strains which then are mated to gain a strain carrying both genes at the same time [74] [75]. This is the reason why the bidirectional approach represents an innovative and promising method to easily express two genes in the same microorganism and even control and tune productivity.

So far no researches were done on bi-directional promoter systems in *P. pastoris.* Though, some studies describe bi-directional promoter systems in *S. cerevisiae*. Counting to the most popular, the $P_{GAL1}|P_{GAL10}$ promoter system is one of the best studied bi-directional promoter system in *S. cerevisiae* [76]. Both genes, GAL1 as well as GAL10 are tightly regulated by certain carbon sources and are essential for metabolizing galactose. The promoters are strongly induced by galactose but repressed on glucose [77] [78]. The major advantage of the $P_{GAL1}|P_{GAL10}$ bi-directional promoter system is the ability of producing two different genes simultaneously at almost identical transcription levels [79, 80]. Overall, currently just a handful of bi-directional promoter systems in *S. cerevisiae* were successfully used for protein expression, see Table 4. As shown, all listed promoter combinations feature strong expression levels, which reflects the need for novel bi-directional promoter systems allowing a beneficial lower and weaker protein expression.

Table 4: Overview of bi-directional promoter systems in S. cerevisiae

Promoter system	Gene Products	Regulation	Expression Level	References
P_{GAL1}|P_{GAL10}	GAL1 = galactokinase 1 GAL10 = galactokinase 10	Inducible by galactose Inducible by galactose	Both strong; fixed ratio 1:1	[79, 81]
P_{GPD}|P_{ADH1}	GPD = glyceraldehyde 3-phosphate dehydrogenase ADH1 = alcoholdehydrogenase 1	Constitutive Inducible	Both strong; fixed ratio 1:1	[70]
P_{TEF1}|P_{PGK1}	TEF1 = Transcriptional elongation factor 1 alpha PGK1 = phosphoglycerate kinase	Constitutive Constitutive	Both strong; fixed ratio 1:1	[81]
P_{GAL1}|P_{GPD}	GAL1 = galactokinase 1 GPD = glyceraldehyde 3-phosphate dehydrogenase	Inducible by galactose Constitutive	Both strong; fixed ratio 1:1	[82]
P_{GAL10}/P_{GPD}	GAL10 = galactokinase 10 GPD = glyceraldehyde 3-phosphate dehydrogenase	Inducible by galactose Constitutive	Both strong; fixed ratio 1:1	[82]

To get the maximum use out of bi-directional promoter systems, it is important to know the specific characteristics and features of both promoters. Only with this knowledge, the optimal protein expression can be achieved. Because of the previously mentioned benefits of bi-directional regulation systems, there is a great interest in incorporating them into different host organisms. Due to the reason that *P. pastoris* can be easily cultivated and is already successfully used for recombinant protein expression, it represents a great host organism for using these novel systems.

Model Protein - *Cellobiohydrolase* (CBH)

The high demand for energy is harmful to the environment but could be reduced by using "green" biofuels derived from renewable resources. Therefore, lignocellolytic enzymes needed for degradation of such resources became increasingly popular over the last few years. These enzymes break down pretreated biomass to simple sugars or starch which is then directly used as substrates for ethanol (biofuel) fermentation. One important enzyme for degradation of celluloses is *cellobiohydrolase* [83]. Since studies showed that *P. pastoris* successfully expresses *cellobiohyrolase II* (CBHII) in high yields, the enzyme was used as model protein to follow the productivity of performed bioprocesses [84].

The following paragraphs describe the enzymatic breakdown of the most abundant polysaccharide in biomass, cellulose. Cellulose consists of β-1,4-linked glucose units which shows crystalline as well as amorphous regions [85, 86]. The crystalline structures are the result of strong intermolecular hydrogen bonds, which represent obstacles for sufficient enzyme-substrate interactions and hamper the hydrolysis of cellulose [87].

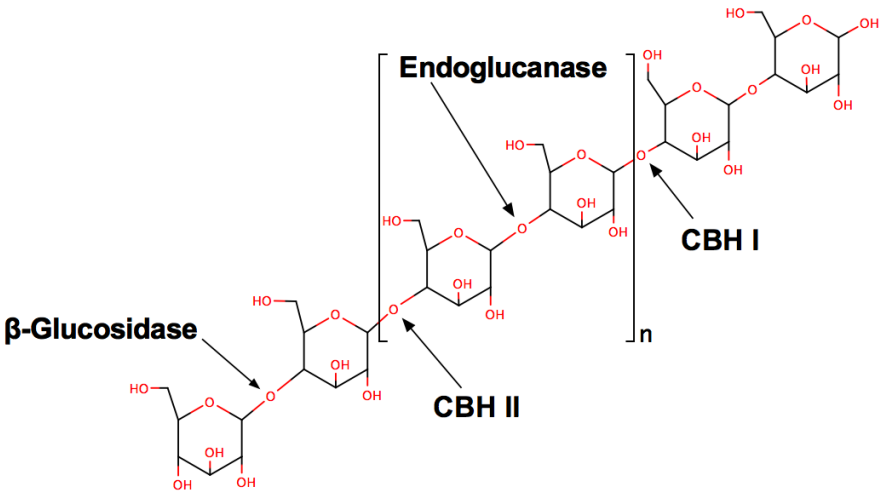

Figure 3: Points of degradation of hydrolytic enzymes; Figure is based on information provided by [87]

Currently paper, food and textile industries are using enzyme mixtures of cellulases derived from *Trichoderma reesei* consisting of exoglucanases, endoglucanases and β-glucosidases (see Figure 3) [84] [87]. The exoglucanases system in *T. reesei* is responsible for the breakdown of crystalline cellulose structures to solubilize it. The system is composed of two different *cellobiohydrolases* (CBHI, CBHII) and they are distinguished into two glycosyl hydrolase families (GH). CBHI belongs to the family of GH7 while CBHII is part of GH6, based on their sequence similarity as well as function and structural connection [87, 88]. Due to the reason

that CBHII mainly reacts on non-reducing ends it differs from CBHI which degrades on reducing ends (see Figure 4) [89, 90].

CBHII as well as CBHI share two common structures, the cellulose-binding module (CBM) and the tunnel shaped catalytic domain for processive degradation [91] [92]. However, CBM is considered to be essential for the breakdown of cellulose since it is responsible for adhering to the polysaccharide chain. Both exoglucanases hydrolyze the β-1,4-linkage of cellulose producing mainly cellobiose based on a general acid catalysis mechanism [93]. The degradation is a stereoselective reaction resulting either in an inverted (CBHII) or retained (CBHI) configuration of the anomeric carbon-atom (C_1) [94] [95].

To conclude, the easy production and wide range of applications make *cellohydrobiolase* II not only an attractive enzyme for the breakdown of lignocellulose, but also good model protein for research.

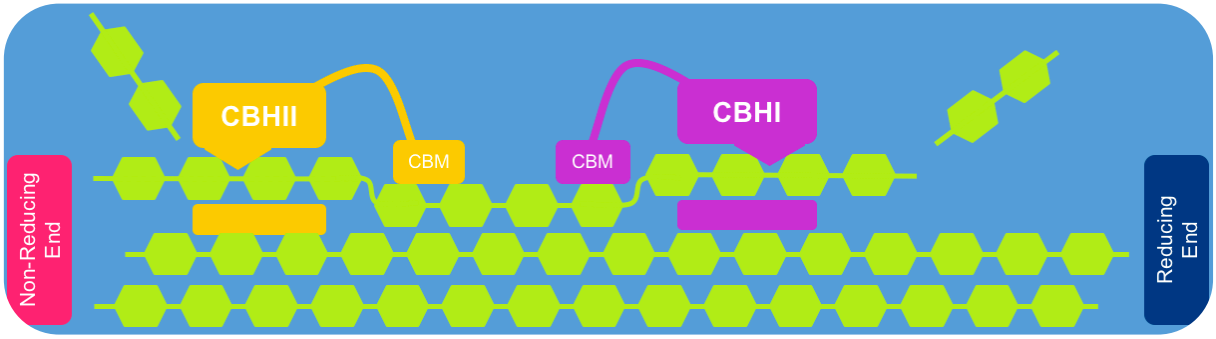

Figure 4: Schematic illustration of cellulose breakdown procedure of CBHI and CBHII; CBM = Carbon-Binding-Module; CBHI or CBHII = catalytic domains

Goals

This Master Thesis relies on two goals:

1. Strain-characterization of *P. pastoris* strain carrying a derepressive and inducible $P_{DC}|P_{AOX1}$ bi-directional promoter system combination
2. Implementation of the novel bi-directional promoter system for tunable and controllable protein production in *P. pastoris* by different feeding strategies

The model protein *cellobiohydrolase II* (CBHII) was expressed to pursue the productivity of the tested cultivation strategies. To reach the above mentioned goals, the challenges were formulated in four scientific questions:

First scientific question - ***Can we successfully cultivate and characterize the novel P. pastoris strain carrying the bi-directional promoter combination $P_{DC}|P_{AOX1}$?***

The strain characterization was performed in the course of glycerol batch cultivations to obtain the main strain specific parameters such as the maximum growth rate (μ_{max}) on glycerol as well as the specific maximum glycerol uptake rate ($q_{S,max,Glycerol}$) and the glycerol biomass yield ($Y_{X/S}$) for developing further cultivation strategies. In the course of a dynamic induction cultivation the maximum specific uptake rate ($q_{S,max,Methanol}$) could be determined.

Second scientific question - ***Is it possible to tune and control recombinant protein production with this novel promoter system?***

To prove if it is possible to activate and to regulate the promoters of the bi-directional promoter system individually, the substrate feeds were changed. The derepressive P_{DC} promoter might be controlled varying the specific glycerol uptake rate ($q_{S,Glycerol}$) while the inducible P_{AOX1} promoter can be induced by methanol.

Third scientific question - ***Is it possible to induce protein production with methanol although glycerol is still present?***

Besides glucose also glycerol causes catabolite repression and prevents the activation of the promoter system and thereby protein expression. To establish a method which allows to use glycerol as well as methanol at the same time to control the promoters concomitantly a dynamic mixed feed strategy was performed.

Fourth scientific question - ***Can we increase productivity by activating both promoter systems?***

To see if the mixed feed strategy shows a significant increase in productivity compared to the derepression- and induction-cultivation phases, a final production cultivation including all three phases was performed. The used specific substrate uptake rates (q_S) for controlling the bioprocess and obtaining a boost in protein production relied on the prior found optimal operation windows.

Structure of Thesis

I answered all mentioned scientific questions in the course of the following paper manuscript. Besides that, additionally performed experiments as well as all occurred problems and used methods will be found in the in the Appendix of my Master Thesis.

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A novel bi-directional promoter system allows tunable recombinant protein production in *Pichia pastoris*

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Abstract

Pichia pastoris has become an important recombinant production host in research and industry. Recombinant protein production in *P. pastoris* is commonly regulated by the methanol inducible P_{AOX} and constitutive P_{GAP} promoter systems. However, both promoter systems are attributed to various disadvantages rendering limited degree of freedom for bioprocess engineers to tune productivity.

A promising and innovative approach to tune and control productivity is the combination of two different promoters with varying characteristics forming a bi-directional promoter system.

In this study, we successfully characterized a recombinant *P. pastoris* strain carrying both a de-repression promoter (P_{DC}) and an inducible promoter (P_{AOX}). We cloned the model enzyme *cellobiohydrolase* II (CBHII) downstream each promoter variant and analyzed how different feeding regimes in the bioreactor activated these promoters and thus productivity. In fact, we were able to tune recombinant protein production using different feeding strategies. We determined optimal operating windows for both promoters to work individually but also concomitantly.

Summarizing, novel bi-directional promoter systems allow tunable recombinant protein production in *P. pastoris* and a high degree of freedom for bioprocess development and optimization.

Keywords: *Pichia pastoris*, bi-directional promoter system, tunable protein expression, strain characterization, fed-batch cultivation, mixed feed

Abbreviations

BSM	Basal salt medium
CBHII	*Cellobiohydrolase II*
c_S	Substrate concentration
c_X	Biomass concentration
DC1	Dynamic cultivation 1
DC2	Dynamic cultivation 2
DC3	Dynamic cultivation 3
DCW	Dry cell weight
dO_2	Dissolved oxygen
FR_0	Initial feed rate
α	Correlation factor
OD_{600}	Optical density at 600 nm
P_{AOX1}	*Alcohol oxidase 1* promoter
P_{AOX2}	*Alcohol oxidase 2* promoter
P_{GAP}	*Glyceraldehyde 3-phosphate dehydrogenase* promoter
P_{GAL1}	*Galactokinase 1* promoter
P_{GAL10}	*Galactokinase 10* promoter
P_{DC}	Modified catalase promoter
$q_{Protein}$	Total protein production rate
$q_{S,Glycerol}$	Specific glycerol uptake rate
$q_{S,MeOH}$	Specific methanol uptake rate
$q_{S,MeOH,Atapt}$	Specific maximum adaptation methanol uptake rate
$q_{S,max,Glycerol}$	Specific maximum glycerol uptake rate
$q_{S,max,MeOH}$	Specific maximum methanol uptake rate
$q_{S,Glycerol,dererpessed}$	Specific glycerol uptake rate at derepressive conditions
rpm	Rounds per minute
V_R	Reactor volume
YNB medium	Yeast nitrogen base medium
$Y_{CO2/S}$	Carbon dioxid yield
$Y_{X/S}$	Biomass yield
μ_{max}	Maximum growth rate

Introduction

The methylotrophic yeast *Pichia pastoris* has become an important host organism for recombinant protein production and is widely used in industrial biotechnology [1-3]. The expression system offers several advantages such as its capability to grow to high cell densities and the availability of performing post-translational modifications. [4-7]. Furthermore, *P. pastoris* can facilitate protein secretion with the help of the alpha mating factor from *Saccharomyces cerevisiae* [4], making it an attractive host for recombinant protein production.

In addition to its capability of using methanol as a sole carbon source, it is usually used to tightly control protein expression through the strong regulated and methanol inducible *alcohol oxidase* promoters (P_{AOX1} and P_{AOX2}) [8, 9]. The decoupling of cell growth and protein production can be seen as a main advantage of these promoters [10]. Even though the P_{AOX} promoters can be precisely controlled, the use of methanol in bioprocesses can cause heat production, increased oxygen demand and might spread into the final product, it is thus a disadvantage [11, 12].

The promoter of the *glyceraldehyde-3-phosphate dehydrogenase* gene (GAP) is another well studied and commonly used expression system in *P. pastoris*, which shows a strong and constitutive behavior. Waterham *et al.* characterized the P_{GAP} promoter and could show significantly higher protein expression levels using P_{GAP} on glucose grown cells compared to methanol grown cells under the control of the P_{AOX1}-promoter [13]. In consideration of P_{GAP} not needing an induction phase, it has become one of the most commonly used promoters [14]. Whereas it has simplified the process of protein production, there also are some downsides to it. The lacking ability of separating cell growth from recombinant protein production can lead to protein folding at a rapid pace resulting in misfolded polypeptides. The same presumably applies to P_{AOX1} [5, 12, 15, 16]. Thus, there is a need for novel, tunable and adjustable systems which allow maximized and controlled protein synthesis. A promising approach is the combination of promoters forming a bi-directional promoter system.
However, state-of-the-art promoter systems in *P. pastoris* such as the P_{AOX1} or the P_{GAP} consist of one promoter directing in one orientation towards a downstream located target gene. In contrast to that, the bi-directional systems are constituted of promoter combinations. These

can vary depending on the desired characteristics of the final system. So far no researches were done using bi-directional promoter systems in *P. pastoris*, though there are some studies describing them in *S. cerevisiae*. $P_{GAL1}|P_{GAL10}$ counts to the most popular systems in this model organism [17]. Both *galactokinase* promoters are induced simultaneously by the same carbon source meaning that the expression of both genes is always connected and happens concomitantly [18, 19]. The goal of combining two promoters forming a bi-directional promoter system with separate controllable gene transcription left us with the demand for suitable options. Previous studies by T. Vogl and A. Glieder [12] described several single promoters of *P. pastoris* for heterologous protein expression with various properties compared to P_{AOX1} and P_{GAP}. This is the foundation on which we could combine different promoters to develop a novel and tunable expression system facing the need of regulation and modulation of protein productivity. Besides that, a major advantage of bi-directional promoter systems is their characteristic to consist of two genes and therefore they possess the ability to produce greater amounts of target protein(s) [20, 21]. The possibility of varying promoter combinations or also target gene combinations allows more freedom for bioprocess design and modulating protein production [22].

In this study we used the promoter combination of $P_{DC}|P_{AOX1}$ which are orientated in opposite directions, as schematically shown in Figure 1. The used modified catalase promoter (P_{DC}) was repressed in presence of glycerol [23, 24]. By reaching a particular derepressing concentration, the promoter was activated and triggered the transcription of the downstream located genes. In addition to that the P_{DC}-promoter was also induced by methanol [25, 26], same applied to the P_{AOX1}-promoter [27], since these promoter options play a key role in the dissimilation of methanol in methylotrophic organisms [28-30].

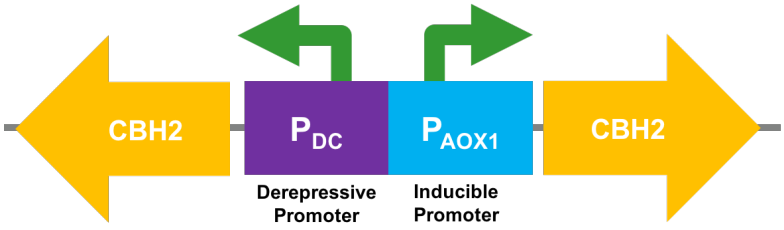

Figure 1: Schematic illustration of bi-directional promoter system of the promoter combination P_{DC} (modified catalase promoter) and P_{AOX1} (alcohol oxidase 1 promoter)

The goals of this study were to make use of the above mentioned promoter features and characterize the *P. pastoris* strain which harbors the derepressive and inducible $P_{DC}|P_{AOX1}$ bi-

directional promoter system. Furthermore, the intention was to implement the novel bi-directional promoter system for tunable protein production in *P. pastoris* by different feeding strategies as well as to maximize the productivity of protein expression.

Material and Methods

Microorganism and model protein

A novel *P. pastoris* strain harboring a bi-directional promoter system (P_{DC} with P_{AOX1}) was provided by Bisy e.U. (Hofstaetten/Raab, Austria).

Studies showed that *P. pastoris* was capable of successfully expressing the lignocellolytic enzyme *cellobiohydrolases II* (CBHII) derived from *Trichoderma reesei* in high yields [31]. Therefore, to monitor the success of the performed cultivations and to follow the productivity, two genes of CBHII were placed downstream each respective promoter option. The genetic modifications were performed by Bisy e.U. (Hofstaetten/Raab, Austria).

The investigated *P. pastoris* strain also carried a zeocin resistance gene inside its plasmid which allowed the cells to withstand the antibiotics even up to concentrations of [100 $\mu g \cdot L^{-1}$].

Cultivation

Bioreactor cultivations

The cultivation of the studied *P. pastoris* strain was carried out in the controlled environment of a bioreactor. A typical bioprocess protocol for *P. pastoris* cells consisted of first a glycerol batch phase for biomass growth, second a glycerol fed-batch to achieve the required biomass and third an induction fed-batch phase [32].

However, the cultivation protocol was modified and adjusted depending on the used (bi-directional) promoter system. In this study the carried out bioprocesses always started with a glycerol batch which was then followed by a customized cultivation phase. The overall workflow of a single cultivation contained the preparation of inoculum followed by the controlled bioprocess in a bioreactor. Offline analytics were performed to evaluate the outcomes.

All bioprocesses were performed in a 5 L lab scale glass fermenter (Infors, Switzerland). The sterilization of the cultivation medium was carried out directly in the bioreactor. During the process different parameters were monitored and controlled online by a process information management system (PIMS, Lucullus, Biospectra, Switzerland). Dissolved oxygen (dO_2) concentration was determined by the help of a fluorescence dissolved oxygen probe (Visiferm

DO425, Hamiltion, Germany) and regulated by stirring and aeration. In case of high cell densities pure oxygen had to be added to the air stream to constantly hold the dO_2 concentration above 30%, controlled by a PID-controller.

The pH was measured with a glass electrode (Easyferm^{TM}, Hamiltion, Switzerland) and adjusted automatically by PIMS using a diluted 12.5% NH_4OH solution. All cultivations were performed at a constant temperature of 30°C and a pH value of 5. Besides the already mentioned parameters, the CO_2 and the O_2 amounts [in %] were measured in the off-gas and recorded for following data evaluation.

The dry cell weight (DCW) concentrations at different time points during the bioprocess were required for controlling. Therefore, the optical density of the cultivation broth at 600 nm (OD_{600}) was measured using a laboratory spectrophotometer (Thermo Scientific Genesys 20, USA). The obtained OD_{600} values were then multiplied by a previous empirically determined proportionality constant (α) to calculate the relative DCW concentrations (see equation 1). This constant describes the correlation between dry cell weight concentrations and the measured OD_{600} values.

$$c_X = OD_{600}\,\alpha$$

equation 1: c_X – calculated biomass dry cell weight concentration [$g_X \cdot L^{-1}$]; OD_{600} – optical density at 600 nm; α – empirical correlation factor between dry cell weight (DCW) and OD_{600}

Precultures

For preculture preparation frozen cryo-samples (-80°C) were cultivated in 100 mL yeast nitrogen base medium (YNB) (as described in studies of Dietzsch *et al.* [33]) supplemented with zeocin. The precultures were carried out in 1000 mL shake flasks to provide enough surface for sufficient oxygen supply.

The cells were incubated at 30°C and at 230 rpm for 24 h. As inoculum-volume 10% of the starting batch-volume was transferred into the bioreactor to start the actual cultivation.

Batch

Biomass production mainly took place during the glycerol batch cultivation on 2-fold basal salt medium (BSM) (recipe based on cultivation protocols of [34]) with a final glycerol concentration of 60 [$g \cdot L^{-1}$]. The end of each batch-cultivation was identified by a rapid decrease of CO_2 in the off-gas signal and a simultaneous increase of dissolved oxygen concentration inside the bioreactor.

Customized cultivation phases

Different specifically designed cultivation phases were carried out to characterize the investigated *P. pastoris* strain harboring the novel bi-directional promoter combination $P_{DC}|P_{AOX1}$ and to tune and control the productivity. This was done by performing customized feeding profiles.

1st dynamic cultivation – DC1

Goal: Determine $q_{S,Glycerol,derepressed}$; $q_{S,max,MeOH}$

The experimental design of the first dynamic cultivation consisted of a glycerol batch followed by a specific glycerol uptake rate ($q_{S,Glycerol}$) controlled dynamic fed batch (derepression phase) and a final dynamic batch with methanol pulses (induction phase).

To activate the inducible P_{AOX1} promoter and to translate the target gene, derepressive conditions and methanol as an inducing agent were required [24, 35]. In general, derepression represents the opposite behavior of repression. In context of the investigated cultivation, it meant the activation of the P_{DC} promoter by crossing a particular glycerol uptake rate ($q_{S,Glycerol,derepressed}$) [23, 24, 35, 36].

The derepression fed batch was performed with decrement controlled $q_{S,Glycerol}$ steps to find the specific glycerol uptake rate where the derepressive P_{DC}-promoter was activated while the P_{AOX1}-promoter was still disabled due to the absence of an inducer. Each step was executed for two hours to attain steady state conditions inside the bioreactor. The feed forward system relied on the glycerol feed based on equation 2 and controlled by PIMS.

$$FR_0 = \frac{c_X V_R}{c_S} q_{S,Glycerol}$$

equation 2: FR_0 – initial feed rate [$L \cdot h^{-1}$]; c_X – calculated biomass wet weight concentration [$g_X \cdot L^{-1}$]; V_R – reactor volume [L]; $q_{S,Glycerol}$ – specific glycerol uptake rate [$g_S \cdot g_X^{-1} \cdot h^{-1}$]; c_S – glycerol feed concentration [$g_S \cdot L^{-1}$]

To investigate the methanol induction behavior of the bi-directional promoter system $P_{DC}|P_{AOX1}$ and to determine the maximum methanol uptake rate ($q_{S,max,MeOH}$) the glycerol fed batch was followed by a dynamic batch with inductive pulses [33, 37]. Therefore, previous to the methanol adaption pulse we waited for the complete glycerol consumption indicated by a decreasing CO_2 off-gas signal. The pulsed methanol was supplemented with 12 [$mL \cdot L^{-1}$] PTM1-trace elements (as described in studies of Dietzsch *et al.* [33]). The adaptation pulse was applied to a final concentration of 0.5% (v/v), for all following pulses 2% (v/v) methanol were injected. To see if temperature had an influence on the maximum methanol uptake rate, the experiments were carried out at two conditions (20°C and 30°C).

2nd dynamic cultivation – DC2

Goal: Find $q_{S,Glycerol}$ where methanol accumulate

This cultivation phase was again appended to a glycerol batch after the entire consumption of glycerol. Before the actual mixed feed phase where glycerol and methanol were fed concomitantly, a 0.5% (v/v) methanol adaptation pulse was applied. The general protocol included a constant methanol feed at $q_{S,max,MeOH}$ and a glycerol feed with increasing $q_{S,Glycerol}$ steps [11, 38]. The aim of the performed cultivation was to determine $q_{S,Glycerol}$ where methanol starts to accumulate caused by the preferred uptake of glycerol [39, 40]. Both, the glycerol feed rate as well as the methanol feed rate were regulated based on the prior mentioned equation and controlled by PIMS.

3rd dynamic cultivation – DC3

Goal: Increase productivity by activating $P_{DC}|P_{AOX1}$

The last cultivation started again with a glycerol batch followed first by a derepression phase, then an induction phase and finally a combined mixed feed phase. That final production cultivation was performed to show the capability to increase productivity even more by activating both promoter options.

The derepression cultivation was carried out at the prior determined $q_{S,Glycerol,derepressed}$ to obtain maximum achievable protein production. That phase was followed by a 0.5 % (v/v) methanol adaptation pulse which enabled the constant methanol feed as the actual induction phase. To show the possibility to increase protein expression a combined mixed feed phase was carried out at prior determined q_S-rates for glycerol as well as for methanol.

Offline analytics

For dry cell weight (DCW) determination 5 mL of cultivation broth were transferred into a 10 mL glass test tube and centrifuged for 10 min at 5°C and 4800 rpm in a laboratory centrifuge (Sigma 3-18K, rotor 11133). After washing the cell pellet with 5 mL deionized water the cells were dried at 105°C for 72h to a constant weight in an oven.

The Bradford Reagent (Product Number: B6916-500ML, Sigma-Aldrich, USA) was used for protein concentration determination at 595 nm in a laboratory spectrophotometer (Thermo Scientific Genesys 20, USA). Bovine serum albumin was used for establishing a standard curve in the range of 0.1 to 1.0 $mg \cdot mL^{-1}$.

Substrate concentrations were determined in the cell free supernatant samples by HPLC measurements (UHPLC, Dionex UltiMate 3000, Thermo Scientific, USA) using an ion-exchange column (Supelcogel C-610H Sigma-Aldrich, USA) and a refractive index detector (RI-101, Shodex, USA). Filtered and degassed 0.1% H_3PO_4 was used as mobile phase at a constant flow rate of 0.5 $mL \cdot min^{-1}$ and a temperature of 30°C.

To check the actual presence of target protein and to get an idea of the purity of the cultivation broth, electrophoresis runs were performed using commercially available SDS-PAGE gels (Amersham™ ECL™ Gel 8-16%). The runs were carried out in a horizontal electrophoresis camber (Amersham™ ECL™ Gel Box, GE, USA) and afterwards stained using Coomassie blue staining solution. The SeeBlue® Plus2 Prestained Standard ladder (Invitrogen, ThermoFisher Scientific, USA) has been used as a protein mass standard.

Results & Discussion

In the course of this study a recombinant *P. pastoris* strain carrying the bi-directional promoter combination $P_{DC}|P_{AOX1}$ was characterized. Different feeding profiles were applied to tune and maximize protein expression. The total protein production rate ($q_{Protein}$) was determined by measuring the total protein content. In addition to that, we monitored the presence of the model protein CBHII as well as the relative purity using SDS-PAGE gels.

The strain-specific physiological parameters such as the glycerol biomass yield ($Y_{X/S}$), the maximum growth rate (μ_{max}) and the maximum glycerol uptake rate ($q_{S,max,Glycerol}$) were determined in the course of glycerol batches. We found a maximum growth rate of μ_{max}= 0.27 [h^{-1}] and a glycerol biomass yield of $Y_{X/S}$ = 0.54 [$g_X \cdot g_S^{-1}$]. Based on these two parameters we calculated the maximum glycerol uptake rate of $q_{S,max,Glycerol}$ = 0.51 [$g_S \cdot g_X^{-1} \cdot h^{-1}$].
Prior to each methanol including experiment, a methanol adaptation pulse of 0.5% (v/v) was performed to familiarize the cells with methanol. We obtained a maximum adaptation methanol uptake rate of $q_{S,MeOH,Atapt}$ = 0.014 [$g_S \cdot g_X^{-1} \cdot h^{-1}$] at a temperature of 30°C during these phases.
The following chapters will outline the performed dynamic experiments to prove (1) the possibility of tunable and controllable recombinant protein production using the novel $P_{DC}|P_{AOX1}$ promoter system, (2) the inducibility of the bi-directional promoter system in presence of glycerol, and (3) the opportunity to increase the productivity by inducing both promoter systems concomitantly.

DC1

The dynamic derepression cultivation was performed in a series of seven decreasing $q_{S,Glycerol}$ controlled steps to find the specific glycerol uptake rate where derepression of the P_{DC} promoter starts to appear. We actually regulated the q_S-controlled feed forward system ranging from $q_{S,Glycerol}$= 0.57 [$g_S \cdot g_X^{-1} \cdot h^{-1}$] to $q_{S,Glycerol}$= 0.05 [$g_S \cdot g_X^{-1} \cdot h^{-1}$].

As shown in Figure 1 and also summarized in Table 1,the highest productivity in the course of the dynamic derepression cultivation was achieved at $q_{S,Glycerol,derepressed}$ = 0.28 [$g_S \cdot g_X^{-1} \cdot h^{-1}$], which was approximately 50 % of the maximum glycerol uptake rate. However, due to the reason that derepression studies using a mutated AOX1 promoter system showed a significant

lower specific glycerol uptake rate (approximately 10% of $q_{S,max,Glycerol}$), we also expected a lower $q_{S,Glycerol,derepressed}$ for the used novel $P_{DC}|P_{AOX1}$ promoter system [11].

Although Vogl *et al.* used a different catalase promoter system, they were able to show that a decreasing glucose concentration resulted in derepression of the promoter at a certain point. However, productivity reached a maximum at a particular glucose concentration [36]. Though these findings cannot be compared directly, we observed a similar trend. While decreasing $q_{S,Glycerol}$, the productivity and thus the specific protein production rate raised due to the beginning derepression of P_{DC}. At approximately 50% of $q_{S,max,Glycerol}$ the productivity reached its climax. This means that the derepression of the investigated strain happened at a 5 fold higher $q_{S,Glycerol}$ in contrast to the already mentioned studies [11]. Since the promoter combination can be used for protein production at higher $q_{S,Glycerol}$ before the promoter will be repressed, it might be beneficial for bioprocesses with a difficult q_S-controlled feeding system.

Afterwards $q_{Protein}$ decreased again, assuming that the substrate was mainly used for maintenance metabolism. Studies on catalase promoters in other expression hosts showed similar regulation mechanisms [41, 42].

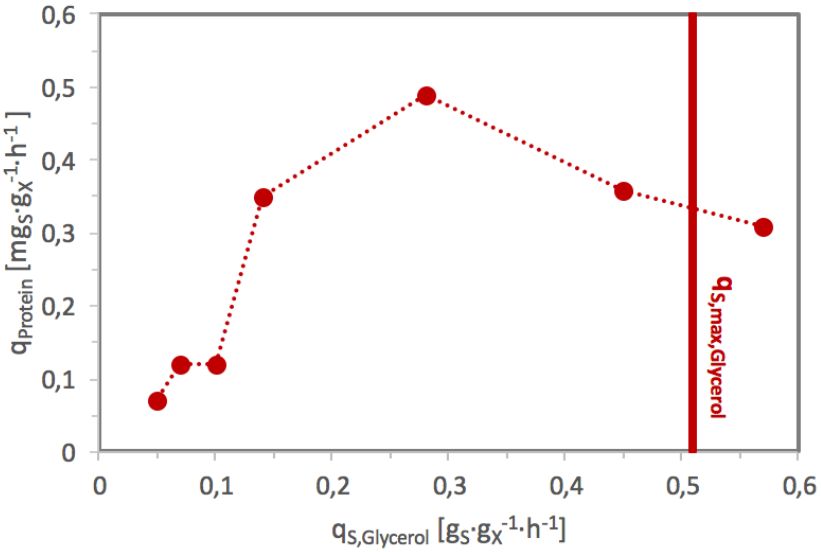

Figure 1: Dynamic derepression cultivation results shown graphically; total protein production rate $q_{Protein}$ [$mg_P \cdot g_X^{-1} \cdot h^{-1}$] plotted against specific glycerol uptake rate $q_{S,Glycerol}$ [$g_S \cdot g_X^{-1} \cdot h^{-1}$]; $q_{S,max,Glycerol}$ indicated by red solid line

Table 1: Summary of derepression cultivation results

DC1 Steps	$q_{S,Glycerol}$ [$g_S \cdot g_X^{-1} \cdot h^{-1}$]	$q_{Protein}$ [$mg_P \cdot g_X^{-1} \cdot h^{-1}$]	$Y_{CO2/S}$ [$Cmol_{CO2} \cdot Cmol_S^{-1}$]	$Y_{X/S}$ [$Cmol_X \cdot Cmol_S^{-1}$]	**C-Balance** [-]
1	0.57	0.31	0.28	0.67	0.95
2	0.45	0.36	0.34	0.67	1.01
3	0.28	0.49	0.37	0.56	0.93
4	0.14	0.35	0.44	0.57	1.01
5	0.10	0.12	0.42	0.50	0.92
6	0.07	0.12	0.55	0.47	1.02
7	0.05	0.07	0.58	0.46	1.04

To find the specific maximum methanol uptake rate $q_{S,max,MeOH}$ and therefore the upper methanol feeding limit, a dynamic batch cultivation with methanol pulses was performed, which represented a fast and easy method to determine physiological parameters [33, 37]. The process was done at two different temperatures by injecting two 2% (v/v) methanol pulses each. In the course of this study we found a significant impact of temperature on $q_{S,max,MeOH}$. At a temperature of 30°C we determined a maximum methanol uptake rate of $q_{S,max,MeOH}$ = 0.025 [$g_S \cdot g_X^{-1} \cdot h^{-1}$] while at 20°C we interestingly obtained a higher value of $q_{S,max,MeOH}$ = 0.029 [$g_S \cdot g_X^{-1} \cdot h^{-1}$]. However, this behavior was also investigated by Gmeiner C. *et al.* using a recombinant *P. pastoris* Δoch1 strain [43]. Nevertheless, other previously performed studies with various microorganisms obtained different results. It was shown that by decreasing temperature, also $q_{S,max,MeOH}$ subsided [44, 45]. So far, there are no explanations for that behavior of the used $P_{DC}|P_{AOX1}$ *P. pastoris* strain.

Based on these findings we postulated the specific maximum methanol uptake rate of $q_{S,max,MeOH}$ = 0.03 [$g_S \cdot g_X^{-1} \cdot h^{-1}$] to provide enough inducing agent during the whole bioprocess for sufficient protein production.

DC2

Based on the performed mixed feed cultivation design of Zalai *et al.* using individually controllable substrate feeds, we developed a mixed feed strategy with a constant methanol feed of $q_{S,max,MeOH}$ = 0.03 [$g_S \cdot g_X^{-1} \cdot h^{-1}$] and a $q_{S,Glycerol}$ controlled glycerol feed which was incremented stepwise [38]. This was done to determine the $q_{S,Glycerol}$ where cell growth and protein production was decoupled. The strategy was premised on the physiological nature of *Pichia* taking up glycerol preferably instead of methanol [39, 40]. By feeding both substrates concomitantly, methanol may accumulate at a certain $q_{S,Glycerol}$ due to the favored glycerol uptake. This will then result in a decoupling of cell growth and protein production due to the starting promoter repression [32].

In the course of the 1st cultivation we observed that the highest productivity and therefore the derepression of the P_{DC} promoter started at $q_{S,Glycerol,derepressed}$ = 0.28 [$g_S \cdot g_X^{-1} \cdot h^{-1}$]. Previous mixed feed studies showed that the highest productivity using a *P. pastoris* Mut^S strain could be achieved at approximately $q_{S,Glycerol}$ = 0.09 [$g_S \cdot g_X^{-1} \cdot h^{-1}$] [38]. Based on these glycerol uptake rates we designed our cultivation having four increasing $q_{S,Glycerol}$ controlled steps which were regulated starting from $q_{S,Glycerol}$ = 0.03 [$g_S \cdot g_X^{-1} \cdot h^{-1}$] up to $q_{S,Glycerol}$ = 0.24 [$g_S \cdot g_X^{-1} \cdot h^{-1}$] (see Table 2).

The biomass yields ($Y_{X/S}$) as well as the protein production rates ($q_{Protein}$) indicated that we were able to decouple biomass growth and protein expression. At the lowest specific glycerol uptake rate and a constant maximum methanol feed rate, the cells starved and showed almost no protein production resulting in a small $q_{Protein}$ and a low biomass yield ($Y_{X/S}$). Increasing $q_{S,Glycerol}$ and regulating $q_{S,MeOH}$ as stable as possible leads to an almost three fold higher productivity, which also shown graphically in Figure 3. By reaching the glycerol uptake rate of $q_{S,Glycerol}$ = 0.24 [$g_S \cdot g_X^{-1} \cdot h^{-1}$] the protein production and thus $q_{Protein}$ dropped. The significant increase of the biomass yield and the decrease of $q_{Protein}$ confirmed the assumption that protein expression stopped and cells mainly focused on cell growth. Other studies reported similar behaviors for *P. pastoris* strains expressing differing proteins [33, 46]. This phenomenon might be explained by exceeding a certain glycerol concentration, the bi-directional promoter system was repressed and thereby the transcription of genes stopped because the cells were preferably using glycerol as energy source [38, 41].

The main outcome of the second cultivation was the postulation of the optimal operation window for concomitant uptake of both substrates. We conclude that the optimal specific glycerol uptake rate, together with a constant methanol feed of $q_{S,max,Methanol}$ = 0.03 [$g_S \cdot g_X^{-1} \cdot h^{-1}$] ranged from $q_{S,Glycerol}$ = 0.06 to 0.14 [$g_S \cdot g_X^{-1} \cdot h^{-1}$]. As shown, the $q_{S,Glycerol}$ setpoint for the mixed feed cultivation compared to the derepression cultivation differs significantly. We assume that high $q_{S,Glycerol}$ values would initiate the shift to using exclusively glycerol for maintenance metabolism, leading to methanol accumulation. Furthermore, the promoter system would be deactivated due to catabolite repression [24, 39, 40].

Table 2: Summary of accumulative cultivation steps

DC2 Steps	**$q_{S,Glycerol}$** [$g_S \cdot g_X^{-1} \cdot h^{-1}$]	**$q_{S,MeOH}$** [$g_S \cdot g_X^{-1} \cdot h^{-1}$]	**$q_{Protein}$** [$mg_P \cdot g_X^{-1} \cdot h^{-1}$]	**$Y_{CO2/S}$** [$Cmol_{CO2} \cdot Cmol_S^{-1}$]	**$Y_{X/S}$** [$Cmol_X \cdot Cmol_S^{-1}$]	**C-Balance** [-]
1	0.03	0.02	0.11	0.81	0.14	0.95
2	0.06	0.03	0.37	0.74	0.17	0.91
3	0.14	0.02	0.35	0.52	0.43	0.95
4	0.24	0.03	0.10	0.44	0.51	0.95

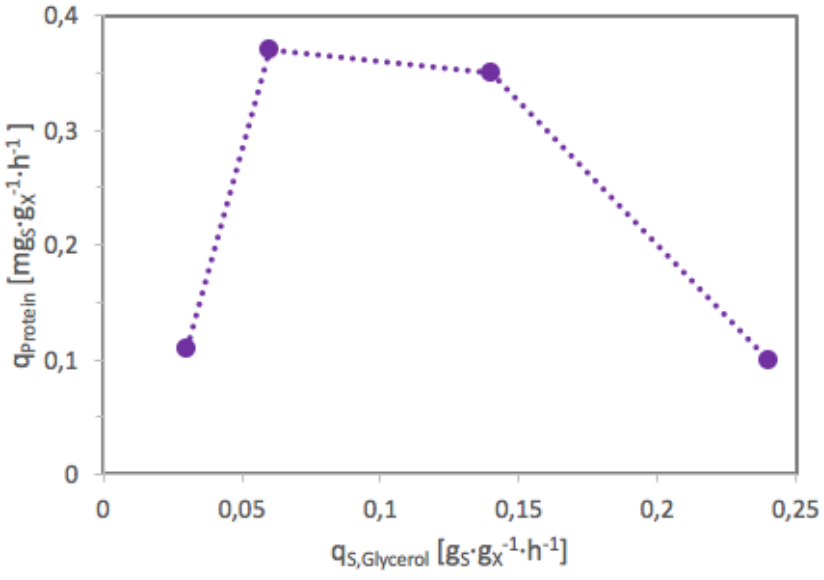

Figure 2: Dynamic mixed feed cultivation results shown graphically; total protein production rate $q_{Protein}$ [$mg_P \cdot g_X^{-1} \cdot h^{-1}$] plotted against specific glycerol uptake rate $q_{S,Glycerol}$ [$g_S \cdot g_X^{-1} \cdot h^{-1}$] at a constant methanol feed of $q_{S,max,MeOH}$ = 0.03 [$g_S \cdot g_X^{-1} \cdot h^{-1}$]

DC3

The final production cultivation was done to carry out all three performed phases - derepression, induction and mixed feed phase - in one single cultivation process. The goal was to prove if it was possible to enhance productivity by deliberately inducing both promoter options of $P_{DC}|P_{AOX1}$ individually or concomitantly.

We previously found the highest specific protein production rate in derepression phase at $q_{S,Glycerol,derepressed}$ = 0.28 [$g_S \cdot g_X^{-1} \cdot h^{-1}$], in induction phase at $q_{S,MeOH}$ = 0.03 [$g_S \cdot g_X^{-1} \cdot h^{-1}$] and in mixed feed phase together with a constant feed of $q_{S,max,MeOH}$ = 0.03 [$g_S \cdot g_X^{-1} \cdot h^{-1}$] at $q_{S,Glycerol}$ = 0.14 [$g_S \cdot g_X^{-1} \cdot h^{-1}$]. Therefore, based on these findings we fixed the particular q_S setpoints for induction phase as well as mixed feed phase at these mentioned substrate uptake rates to gain the highest productivity. To avoid oxygen limitations due to strong biomass growth on glycerol [5, 10], the derepression phase was performed at a lower specific glycerol uptake rate of $q_{S,Glycerol}$ = 0.21 [$g_S \cdot g_X^{-1} \cdot h^{-1}$]. Each production phase was performed for 3 hours to reach steady state conditions for optimal protein expression.

The dynamic derepression experiment was actually regulated at $q_{S,Glycerol}$ = 0.21 [$g_S \cdot g_X^{-1} \cdot h^{-1}$]. We gained a specific protein production rate of $q_{Protein}$ = 0.37 [$mg_P \cdot g_X^{-1} \cdot h^{-1}$] during this phase by solely activating the derepressive P_{DC}-promoter in derepressed conditions. By comparing the results to the outcome of the 1st cultivation it can be seen that the findings were similar and therefore reproducible.

The followed induction phase was controlled at the maximum specific methanol uptake rate of $q_{S,MeOH}$ = 0.03 [$g_S \cdot g_X^{-1} \cdot h^{-1}$] which resulted in an approximate 5-fold lower total protein production rate of $q_{Protein}$ = 0.07 [$mg_P \cdot g_X^{-1} \cdot h^{-1}$] compared to the prior derepression phase. Studies describe catalase as well as alcohol oxidase promoters as strong and tightly regulated inducible promoter options in *P. pastoris* [9, 12, 36]. This lead to the assumption that the induction phase would result in a higher $q_{Protein}$ compared to the derepression phase. However, the obtained outcomes are contradictory. There is no evidence yet regarding the reasons, although we hypothesize that the used bi-directional promoter combination is not as strongly inducible by methanol as assumed.

The combination of induction- with derepression phase resulted in the last part of the final production cultivation, the mixed feed phase. In respect of productivity, the optimal operating

window could be determined in the course of the dynamic mixed feed cultivation. The required glycerol feed was accordingly controlled at $q_{S,Glycerol}$ = 0.14 [$g_S \cdot g_X^{-1} \cdot h^{-1}$] while the methanol feed resulted in a specific methanol uptake rate of $q_{S,MeOH}$ = 0.04 [$g_S \cdot g_X^{-1} \cdot h^{-1}$]. As shown in Table 3 the mixed feed phase reached an approximately 1.5-fold higher $q_{Protein}$ of $q_{Protein}$ = 0.57 [$mg_P \cdot g_X^{-1} \cdot h^{-1}$] compared to the derepression phase. However, HPLC analysis revealed that small amounts of methanol accumulated right at the beginning of the cultivation phase, which was then consumed throughout the mixed feed step. This finding might be explained by the reinstated glycerol feed causing a preferred uptake of glycerol compared to methanol. It seemed that the cells required some time to adapt to the availability of two different substrates before they were able to use these carbon sources concomitantly. This assumption was confirmed by the decreasing methanol concentration over time.

Table 3: Summary of final production cultivation

DC3 Phases	**$q_{S,Glycerol}$** [$g_S \cdot g_X^{-1} \cdot h^{-1}$]	**$q_{S,MeOH}$** [$g_S \cdot g_X^{-1} \cdot h^{-1}$]	**$q_{Protein}$** [$mg_P \cdot g_X^{-1} \cdot h^{-1}$]	**$Y_{CO2/S}$** [$Cmol_{CO2} \cdot Cmol_S^{-1}$]	**$Y_{X/S}$** [$Cmol_X \cdot Cmol_S^{-1}$]	**C-Balance** [-]
Derepression	0.21	-	0.37	0.60	0.48	1.08
Induction	-	0.03	0.07	0.80	0.10	0.90
Mixed Feed	0.14	0.04	0.57	0.55	0.53	1.08

State-of-the art techniques for heterologous protein production in *P. pastoris* are the usage of single promoters. Depending on their certain characteristics they are controlled either by derepression or induction. Studies showed that *Pichia's* inducible single promoter systems among derepression promoters are better established and due to their strong and tight regulation they yield in high productivity [12, 30, 47, 48]. However, we could demonstrate that the novel bi-directional promoter system achieved a considerably higher $q_{Protein}$ solely in the derepression fed batch compared to the induction cultivation. Though, we obtained an even higher productivity in the combined mixed phase, which resulted in an almost 1.5-fold higher total specific protein production rate in comparison to the derepression phase (see Table 3). These results confirmed the many benefits of using bi-directional promoter combinations for increased protein expression and the need to further investigate such promising systems. Especially for the biopharmaceutical industry it is particularly promising to be able to regulate the production of two proteins in high amounts in a single microorganism.

Generally speaking, bi-directional promoter systems represent a good and profitable expression system and are expected to simplify and optimize bioprocesses.

Conclusion

In the present study we successfully characterized the *P. pastoris* strain carrying the bi-directional promoter system of P_{DC}/P_{AOX1}. Our findings can be summed up:

- We were able to find both, the specific maximum glycerol uptake rate as well as the specific maximum methanol uptake rate for the investigated strain. Besides these important parameters also the glycerol uptake rate $q_{S,Glycerol,derepressed}$ where the P_{DC}-promoter is derepressed, could be found. This allowed the development of the mixed feed design.
- Furthermore, we delivered the optimal operating window for mixed feed cultivation in respect of the highest protein productivity. It was shown that the promoters worked individually or concomitantly using different feeding strategies.
- The final production cultivation included all three previously performed cultivation phases in one single bioprocess, resulting in the highest productivity in the mixed feed phase where both promoters of the bi-directional promoter system were simultaneously active.

Currently we are working on substituting methanol with oleic acid to avoid handling the highly flammable and hazardous substrate. In addition, other novel bi-directional promoter combinations will be tested using the so far discovered findings to develop a standard operation protocol. However, the present study represents a good basis for strain characterization and designing new bioprocesses using bi-directional promoter systems in *P. pastoris* as well as a possible method to increase productivity using a mixed feed strategy.

Appendix

DC1

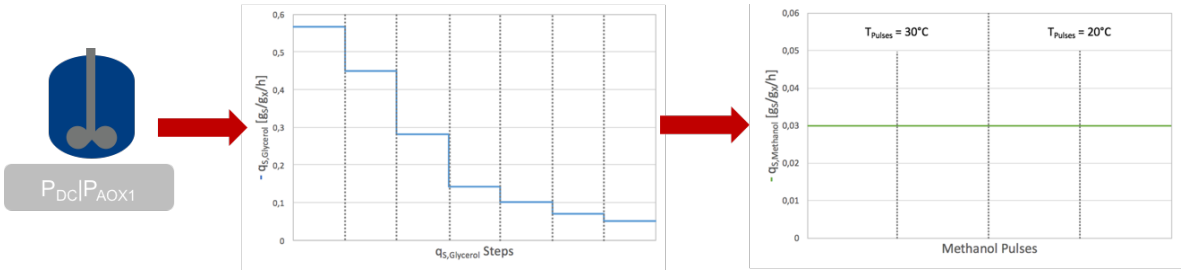

Figure 4: Schematic illustration of dynamic cultivation DC1; glycerol fed batch → glycerol derepression fed batch → dynamic methanol pulse experiment

DC2

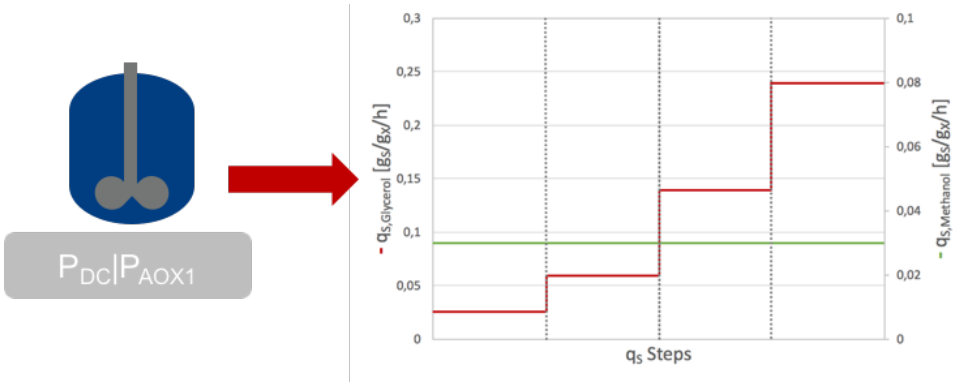

Figure 5: Schematic illustration of dynamic cultivation DC2; glycerol fed batch → dynamic mixed feed cultivation

DC3

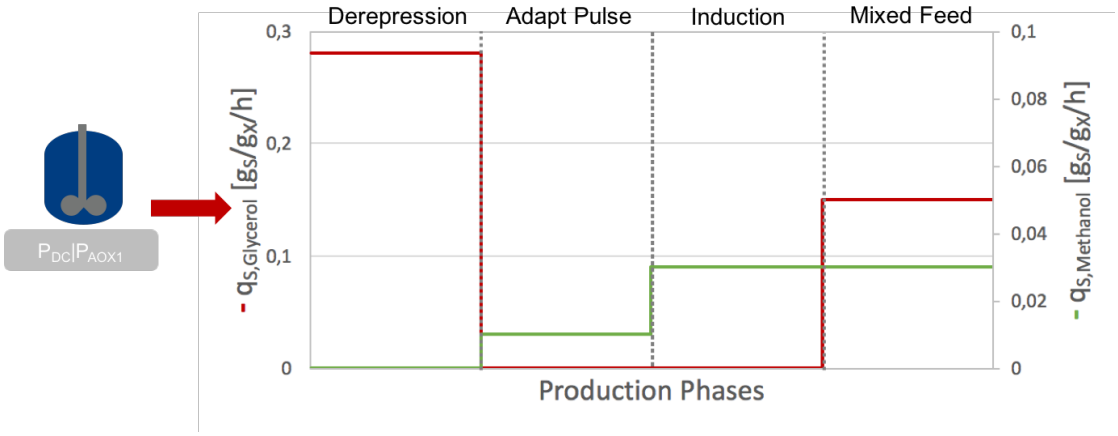

Figure 6: Schematic illustration of dynamic cultivation DC3; glycerol fed batch → glycerol derepression fed batch → methanol adaptation pulse → methanol induction fed batch → production mixed feed cultivation

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Conclusion

In the course of my Master Thesis I was able to characterize the *P. pastoris* strain carrying the derepressive and inducible $P_{DC}|P_{AOX1}$ bi-directional promoter system. Furthermore, I successfully implemented the novel bi-directional promoter system to control and to tune the productivity by changing various feeding strategies. Since no researches have been done or published on bi-directional promoter systems in *P. pastoris* so far, this Thesis provides a novel and innovative strategy to tune and regulate such promoter combinations for achieving high protein expression. Below I want to answer to the scientifc questions raised at the beginning of my Thesis.

First scientific question - ***Can we successfully cultivate and characterize the novel P. pastoris strain carrying the bi-directional promoter combination $P_{DC}|P_{AOX1}$?***

Already the first glycerol batch cultivation showed that the novel *P. pastoris* strain was able to be cultivated in a defined and controlled 5L lab scale bioreactor. All subsequently performed cultivations delivered similar outcomes resulting in an averaged maximum growth rate on glycerol of μ_{max} = 0.27 [h^{-1}], an averaged biomass yield on glycerol of $Y_{X/S}$ = 0.54 [$g_X \cdot g_S^{-1}$] and an averaged maximum glycerol uptake rate of $q_{S,max,Glycerol}$ of 0.51 [$g_S \cdot g_X^{-1} \cdot h^{-1}$]. The dynamic induction cultivation at two different temperatures delivered the highest productivity at 30°C at $q_{S,MeOH}$ = 0.025 [$g_S \cdot g_X^{-1} \cdot h^{-1}$] [1, 2].

Main outcome:

All carried out bioprocesses showed similar strain specific parameters concluding that the cultivations as well as the strain characterization were successful.

Second scientific question - ***Is it possible to tune and control recombinant protein production with this novel promoter system?***

The results of the combined derepression and dynamic induction cultivation showed that it was possible to modulate protein production by differing feeding strategies. Each promoter option of the novel bi-directional promoter combination could be individually activated. The performed glycerol fed batch, controlled by decreasing $q_{S,Glycerol}$ steps, fully activated the derepressive P_{DC}-promoter at about $q_{S,Glycerol,derepressed}$ = 0.28 [$g_S \cdot g_X^{-1} \cdot h^{-1}$] indicated by a significant increase of the specific total protein production rate.

Figure 1 as well as Figure 2 illustrate schematically the activation of each particular promoter option under derepressive or inducible conditions. Due to the reason that the P_{DC}-promoter can also be slightly activated by methanol, a thin arrow indicates the weak transcription of the *cbhII*-gene under inducible conditions. However, it resulted in a lower $q_{Protein}$ compared to the derepression phase.

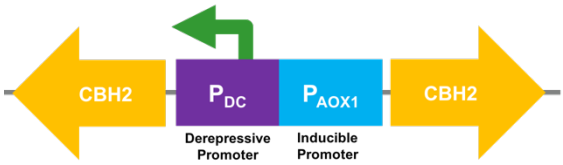


Figure 1: Schematic illustration of $P_{DC}|P_{AOX1}$ bi-directional promoter system under derepressive conditions

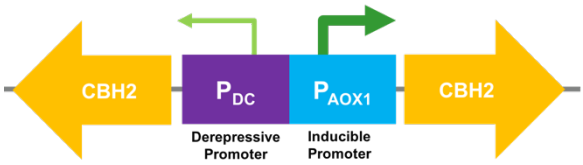


Figure 2: Schematic illustration of $P_{DC}|P_{AOX1}$ bi-directional promoter system under inducible conditions

Main outcome:
By changing the feeding strategies in the course of the bioprocess it was possible to regulate and to control protein expression by activating the desired promoter option.

Third scientific question - *Is it possible to induce protein production with methanol although glycerol is still present?*

The dynamic mixed feed cultivation was performed, to prove if the *P. pastoris* strain carrying the $P_{DC}|P_{AOX1}$ promoter combination would be able to concomitantly uptake methanol and glycerol [3]. Meaning that methanol was fed constantly at $q_{S,max,MeOH} = 0.030$ [$g_S \cdot g_X^{-1} \cdot h^{-1}$] while glycerol was added by controlled increasing $q_{S,Glycerol}$ steps to find the glycerol uptake rate where glycerol preferably would be metabolized and methanol would accumulate [4, 5]. By performing this special form of mixed feed experiment, the most productive operation window could be determined and ranged from $q_{S,Glycerol} = 0.06$ [$g_S \cdot g_X^{-1} \cdot h^{-1}$] to $q_{S,Glycerol} = 0.14$ [$g_S \cdot g_X^{-1} \cdot h^{-1}$] at a constant $q_{S,max,MeOH} = 0.030$ [$g_S \cdot g_X^{-1} \cdot h^{-1}$]. This implies that the methanol induction of the P_{AOX1}-promoter, as well as the activation of the P_{DC}-promoter was able to happen at the same time. Compared to the derepression phase it occurred at rather low specific glycerol uptake rates. Figure 3 schematically shows the active bi-directional promoter combination under mixed feed conditions, resulting in two transcriptionally active promoters.

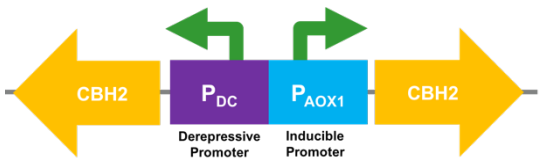


Figure 3: Schematic illustration of $P_{DC}|P_{AOX1}$ bi-directional promoter system under mixed feed conditions

Main outcome:
The experiment was able to show the activation of both promoter options using methanol as well as glycerol at the same time. In addition to that, the optimal operation window for mixed feed cultivation could be determined.

Fourth scientific question - *Can we increase productivity by activating both promoter systems?*

In the course of the final production cultivation all three cultivations phases - derepression, induction and mixed feed - were carried out successfully. Based on the previous found q_S set points, which resulted in highest productivity for each respective cultivation phase, the process design for the final production cultivation was developed. The actual results of this experiment are summarized graphically in Figure 4. As shown, the induction phase at $q_{S,MeOH}$ = 0.03 [$g_S \cdot g_X^{-1} \cdot h^{-1}$] phase resulted in the lowest protein production rate ($q_{Protein}$). The derepression phase regulated at $q_{S,Glycerol}$ = 0.21 [$g_S \cdot g_X^{-1} \cdot h^{-1}$] achieved an almost 5 fold higher productivity compared to the induction phase. However, the protein expression could be even further increased by feeding methanol at $q_{S,MeOH}$ = 0.04 [$g_S \cdot g_X^{-1} \cdot h^{-}1$] and glycerol at $q_{S,\ Glycerol}$ = 0.14 [$g_S \cdot g_X^{-1} \cdot h^{-1}$] in a mixed feed strategy concomitantly. It reached the highest productivity of all performed phases.

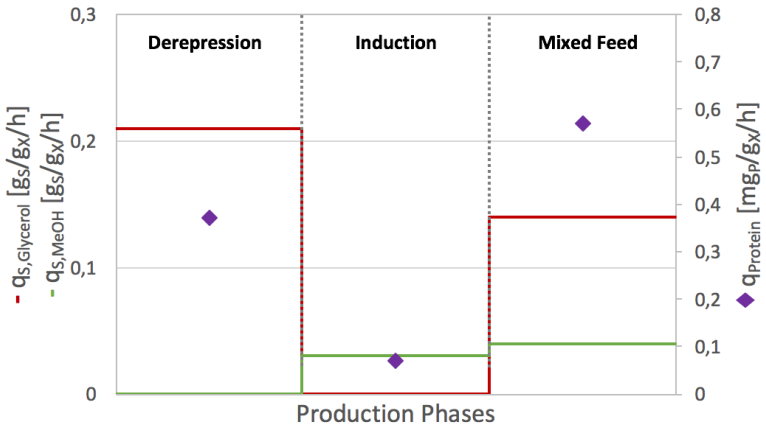


Figure 4: Results of final production cultivation summed up graphically; red curve: actual controlled specific glycerol uptake rate $q_{S,Glycerol}$ [$g_S \cdot g_X^{-1} \cdot h^{-1}$]; green curve: actual controlled specific methanol uptake rate $q_{S,Glycerol}$ [$g_S \cdot g_X^{-1} \cdot h^{-1}$]; purple diamond: specific total protein production rate $q_{Protein}$ [$mg_P \cdot g_X^{-1} \cdot h^{-1}$]

Main outcome:

It was possible to enhance productivity by deliberately activating both promoter options of $P_{DC}|P_{AOX1}$ concomitantly.

Appendix

Additional experiments and results – DASbox® Cultivations

In addition to the strain characterization of the *P. pastoris* strain carrying the $P_{DC}|P_{AOX1}$ bi-directional promoter system, two further promoter combinations were characterized. However, the promoter systems $P_{DC}|P_{DF}$ and $P_{DF}|P_{GAP}$ were not cultivated using the 5L labfors glass fermenter system, instead the strains were screened using the Eppendorf DASbox® Mini Bioreactor system [6]. This method contains four 350 mL glass vessels allowing parallel cultivation (see Figure 5). Although the working volume (60 – 250 mL) is rather small, all important process parameters such as pH, temperature, dissolved oxygen, OD as well as the off gas composition can be measured and monitored. By using an efficient soft-sensor it is even possible to directly control the bioprocess. Nevertheless, one mentionable limitation of the used system was the missing ability to use two substrate feeds at the same time since it only includes one single substrate pump per reactor. This has to be considered for bioprocess development.

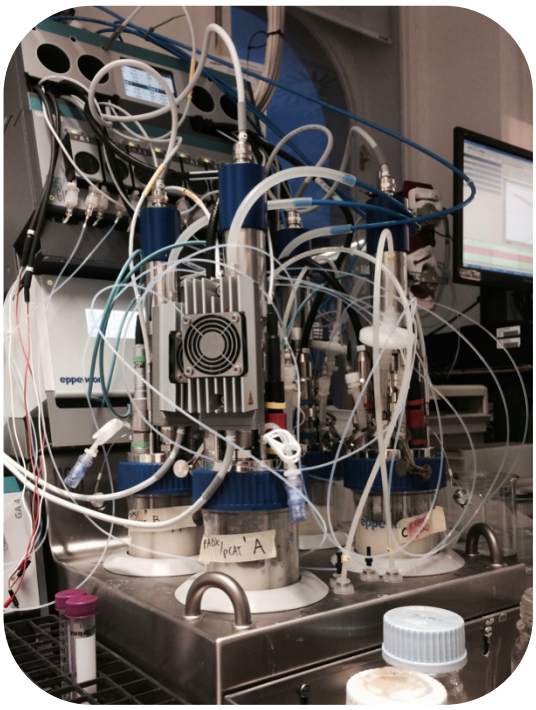


Figure 5: Eppendorf DASbox® Mini Bioreactor System with four vessels

Due to this mini bioreactor system enabling a completely controlled cultivation process, it has been used to cultivate all three bi-directional promoter combinations ($P_{DC}|P_{AOX1}$ and $P_{DC}|P_{DF}$ and $P_{DF}|P_{GAP}$) simultaneously.

Microorganism and Model Protein

As already mentioned, overall three different bi-directional promoters were used for further experiments which were all carried out in the Eppendorf DASbox® Mini Bioreactor System (see Table 1): the already characterized $P_{DC}|P_{AOX1}$ combination and two new promoter systems the $P_{DC}|P_{DF}$ and $P_{DF}|P_{GAP}$. All strains were provided by Prof. Anton Glieder (Bisy, Austria). Also *cellobiohydrolases II* (CBHII) of *Trichoderma reesei* was used as a model protein to follow the productivity as well as the efficiency of the investigated promoter combinations.

Table 1: Overview of used and characterized bi-directional promoter systems and their respective gene products.

Promoter system	Gene Products	Regulation
P_{DC}/P_{AOX1}	DC = modified catalase	Derepression and Inducible by methanol
	AOX1 = alcohol oxidase 1	Inducible by methanol
P_{DC}/P_{DF}	DC = modified catalase	Derepression and Inducible by methanol
	DF = modified formaldehyde dehydrogenase	Derepression and Inducible by methanol and methylamine
P_{DF}/P_{GAP}	DF = modified formaldehyde dehydrogenase	Derepression and Inducible by methanol and methylamine
	GAP = glyceraldehyde 3-phosphate dehydrogenase	Constitutive

Goals of the Eppendorf DASbox® Mini Bioreactor System

Since this system was not well established in our lab and no cultivations had been performed in such small scales before, the goals of this experiment were formulated straightforward.

- Validation of strain characteristic parameters of the $P_{DC}|P_{AOX1}$ *P. pastoris* strain found in course of the 5L scale cultivations
- Strain characterization of both new *P. pastoris* strains carrying the $P_{DC}|P_{DF}$ and the $P_{DF}|P_{GAP}$ bi-directional promoter system combination

Cultivation strategy

Precultures were prepared as previously described in the paper draft using YNB medium. Batch cultivation phases with 2-fold basal salt medium were carried out as well, but with lower initial glycerol concentration. Instead of 60 [$g \cdot L^{-1}$] glycerol only 30 [$g \cdot L^{-1}$] was used, due to the reason that the available optical density-probes for biomass concentration determination were solely usable until a particular biomass concentration.

Overall, two different cultivation runs were performed using four mini reactors at the same time. Each bioprocess followed the same workflow schematically illustrated in Figure 6.

Figure 6: General process workflow of performed DASbox® cultivations

First DASbox® Cultivation - Strategy

The glycerol batch phase was followed by a $q_{S,Glycerol}$ controlled fed batch phase to validate the already found physiological parameters for the $P_{DC}|P_{AOX1}$ strain. In addition, the goal was to determine strain specific parameters as well as to find $q_{S,Glycerol,derepressed}$ for the other two alternative promoter combinations. Therefore, for all three recombinant *P. pastoris* strains the derepression fed batch was carried out by stepwise decreasing $q_{S,Glycerol}$ to cross a particular threshold where the derepressive promoter options are activated resulting in a higher specific protein production rate ($q_{Protein}$). Since no physiological data for the promoter systems $P_{DC}|P_{DF}$ and $P_{DF}|P_{GAP}$ where available, the already determined maximum glycerol uptake rate of $P_{DC}|P_{AOX1}$ was also used for the cultivation design. Overall four $q_{S,Glycerol}$ steps were performed starting at 50% of $q_{S,max,Glycerol}$ going down to to 5% of $q_{S,max,Glycerol}$ (see Figure 7).

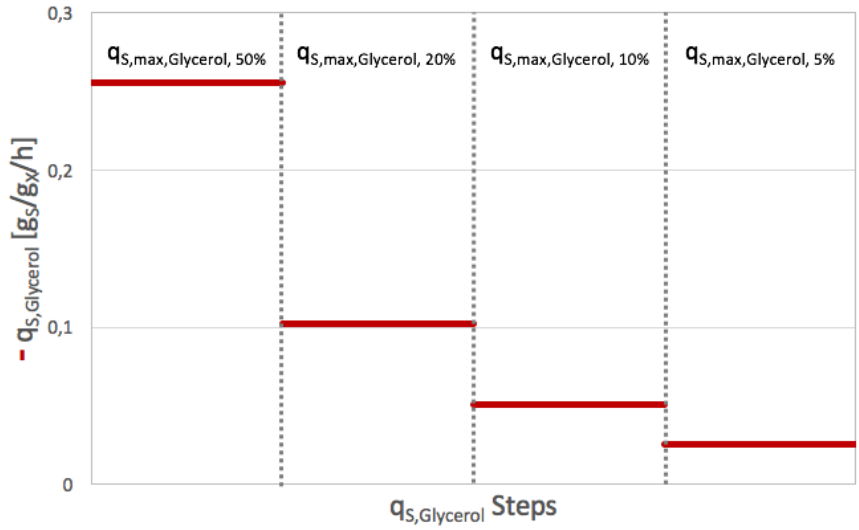


Figure 7: Schematic diagram of $q_{S,Glycerol}$ set points of derepression fed

First DASbox® Cultivation - Results

The different *P. pastoris* strains carrying various bi-directional promoter systems were cultivated successfully using the Eppendorf DASbox® Mini Bioreactor system. Although the bioprocess was performed under sterile conditions one out of four bioreactors (the backup $P_{DC}|P_{AOX1}$ strain) showed contaminations and was therefore neglected for further data evaluation.

It was also possible to characterize the so far unknown strains as well as to validate the already determined specific strain parameters for the $P_{DC}|P_{AOX1}$ promoter combination. As shown in Table 2 the obtained values for μ_{max}, $q_{S,max,Glycerol}$ and $Y_{X/S}$ can be considered similar.

Determined parameters for the two residual promoter combinations are summarized in Table 3. The data validity was confirmed by closing C-balances.

Table 2: Comparison of strain specific parameters of P. pastoris carrying $P_{DC}|P_{AOX1}$ determined from two seperate performed cultivations; values of Master Thesis Cultivation were averaged

		Master Thesis Cultivation	DASbox® Cultivation
Strain specific parameters		**$P_{DC}|P_{AOX1}$**	**$P_{DC}|P_{AOX1}$**
μ_{max}	$[h^{-1}]$	0.27	0.28
$q_{S,max,Glycerol}$	$[g_S \cdot g_X^{-1} \cdot h^{-1}]$	0.51	0.53
$Y_{X/S}$	$[g_X \cdot g_S^{-1}]$	0.54	0.54
C-Balance	[-]	1.10	1.02

Table 3: Strain specific parameters of P. pastoris strain carrying $P_{DC}|P_{DF}$ and $P_{DF}|P_{GAP}$

		DASbox® Cultivation	
Strain specific parameters		**$P_{DC}|P_{DF}$**	**$P_{DF}|P_{GAP}$**
μ_{max}	$[h^{-1}]$	0.20	0.21
$q_{S,max,Glycerol}$	$[g_S \cdot g_X^{-1} \cdot h^{-1}]$	0.37	0.34
$Y_{X/S}$	$[g_X \cdot g_S^{-1}]$	0.55	0.61
C-Balance	[-]	0.92	1.05

However, the previous determined $q_{S,Glycerol,derepressed}$ could not be found for the $P_{DC}|P_{AOX1}$ promoter combination, on the grounds of the $q_{S,Glycerol}$ steps being controlled at too low glycerol rates. Most important to mention is that the specific protein production rates ($q_{Protein}$) could not be affirmed, since the obtained values were significantly higher compared to previous cultivations (see Table 4). An explanation might be found in the dissolved oxygen (dO_2) data of the processes, because in all three cases the dO_2-concentrations oscillated immensely resulting in stressful hypoxic condition for cells. Studies by Baumann K. and Maurer M. *et al.* showed that oxygen deficiency in *P. pastoris* caused an increase in specific protein production rates [7]. Therefore, it can be assumed that hypoxic conditions were the reason for the occurred high protein production in our processes. Nevertheless, although the absolute values were not comparable, the trends in $q_{Protein}$ were still considerable.

In Table 4 all obtained results of the derepression experiment for each bi-directional promoter combination were summarized. As shown in Figure 8 as well, the *P. pastoris* strain carrying the $P_{DC}|P_{AOX1}$ promoter combination resulted in the lowest specific total protein production rates compared to both other strains. It consisted of one derepressive and one solely methanol inducible promoter [8-10]. Due to the reason that the P_{AOX1} promoter was repressed in the presence of glycerol, leaving only the other one active, the $P_{DC}|P_{AOX1}$ combination resulted in the lowest productivity.

At low $q_{S,Glycerol}$ rates the $P_{DC}|P_{DF}$ promoter system showed slightly higher production rates in contrast to $P_{DF}|P_{GAP}$. Nevertheless, at higher specific glycerol uptake rates the $P_{DF}|P_{GAP}$ combination showed an almost 1.5 fold higher productivity. Since $P_{DC}|P_{DF}$ promoter combination was composed of two derepressive promoter options, it could be activated by reaching derepressive conditions at presumably low glycerol uptake rates.

When glycerol is available P_{GAP} is permanently active since it is a constitutive promoter [11-13]. Because of its strong production behavior, the derepressive/constitutive $P_{DC}|P_{GAP}$ system outperformed and presented the highest productivity of all three investigated strains.

However, to confirm these findings and to determine the absolute values, a controlled bioprocess in a 5L scale needed to be performed.

Table 4: Extract of results of performed Master Thesis Cultivation and summarized results of DASbox® derepression cultivation for the bi-directional promoter combinations $P_{DC}|P_{AOX1}$, $P_{DC}|P_{DF}$ and $P_{DF}|P_{GAP}$; cells highlighted in gree = full derepression

Master Thesis Cultivation		**DASbox® cultivation**					
$P_{DC}|P_{AOX1}$		**$P_{DC}|P_{AOX1}$**		**$P_{DC}|P_{AOX1}$**		**$P_{DC}|P_{AOX1}$**	
$q_{S,Glycerol}$	$q_{Protein}$	$q_{S,Glycerol}$	$q_{Protein}$	$q_{S,Glycerol}$	$q_{Protein}$	$q_{S,Glycerol}$	$q_{Protein}$
$[g_S \cdot g_X^{-1} \cdot h^{-1}]$	$[mg_P \cdot g_X^{-1} \cdot h^{-1}]$	$[g_S \cdot g_X^{-1} \cdot h^{-1}]$	$[mg_P \cdot g_X^{-1} \cdot h^{-1}]$	$[g_S \cdot g_X^{-1} \cdot h^{-1}]$	$[mg_P \cdot g_X^{-1} \cdot h^{-1}]$	$[g_S \cdot g_X^{-1} \cdot h^{-1}]$	$[mg_P \cdot g_X^{-1} \cdot h^{-1}]$
0.14	0.35	0.18	0.54	0.18	0.95	0.20	1.40
0.10	0.12	0.07	0.22	0.06	1.11	0.07	0.98
0.07	0.12	0.03	0.29	**0.03**	**1.20**	0.04	1.00
0.05	0.07	0.02	0.17	0.01	0.56	0.02	0.50

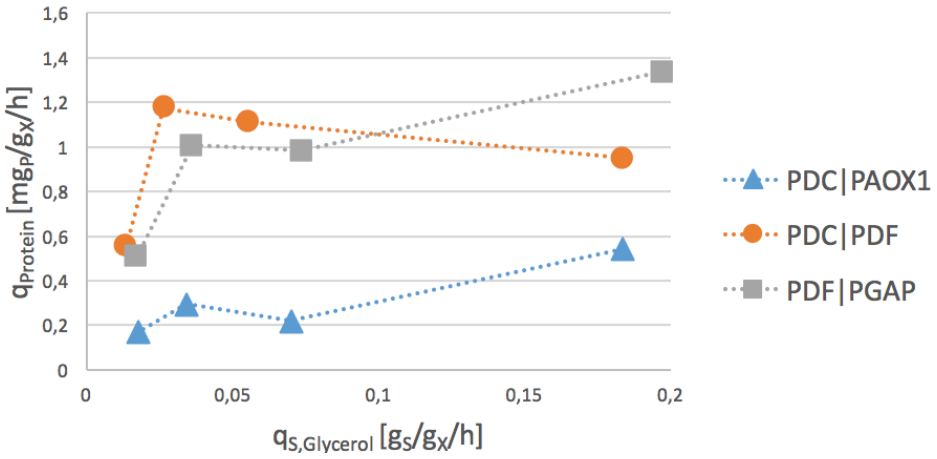


Figure 8: Results of first DASbox® cultivation summarized in a $q_{Protein}$ vs. $q_{S,Glycerol}$ plot to see optimal operating window

Second DASbox® Cultivation - Strategy

The second performed cultivation differed from the first cultivation regarding the used substrate as well as the customized cultivation phase. Right after the batch, a glycerol fed batch was performed to gain additional biomass since no biomass would be gained during the subsequent methanol adaptation pulse as well as the methanol induction fed batch. The induction phase was designed in three increasing $q_{S,MeOH}$ controlled steps starting from 50% of $q_{S,max,MeOH}$ up to 150% of $q_{S,max,MeOH}$. The maximum methanol uptake rate had been found in previous cultivations of $P_{DC}|P_{AOX1}$. Since no reference data about $q_{S,max,MeOH}$ for the promoter combinations $P_{DC}|P_{DF}$ and the $P_{DF}|P_{GAP}$ were available, the same $q_{S,max,MeOH}$ derived from $P_{DC}|P_{AOX1}$ had been used. A schematic diagram presenting the controlled $q_{S,MeOH}$ steps is shown in Figure 9. The goal of the second DASbox® cultivation was to find the methanol uptake limit of the cells indicated by the accumulation of the substrate. Furthermore, it was interesting to investigate the effect of exceeding $q_{S,max,MeOH}$ on the specific total protein production rate.

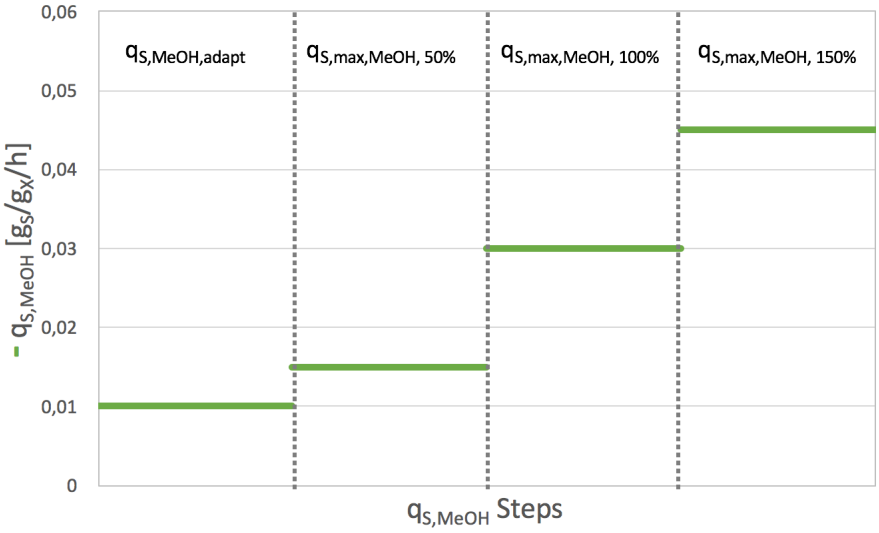


Figure 9: Schematic diagram of controlled $q_{S,Methanol}$ induction fedbatch

Second DASbox® Cultivation – Results

The glycerol batch performed prior to the customized cultivation phases delivered following results, summarized in Table 5. By comparing these outcomes with the found strain specific parameters of the first DASbox® cultivation, it is shown that the values slightly distinguished. However, closing C-balances validated these data and thus they could be considered as similar.

Table 5: Summarized glycerol batch results of obtained strain specific parameters for the bi-directional promoter combinations $P_{DC}|P_{AOX1}$, $P_{DC}|P_{DF}$ and $P_{DF}|P_{GAP}$

Glycerol Batch		**DASbox® Cultivation**		
Strain specific parameters		**$P_{DC}|P_{AOX1}$**	**$P_{DC}|P_{DF}$**	**$P_{DF}|P_{GAP}$**
μ_{max}	$[h^{-1}]$	0.24	0.23	0.22
$q_{S,max,Glycerol}$	$[g_S \cdot g_X^{-1} \cdot h^{-1}]$	0.42	0.43	0.40
$Y_{X/S}$	$[g_X \cdot g_S^{-1}]$	0.58	0.53	0.55
C-Balance	[-]	0.98	0.93	0.97

After the complete glycerol depletion of the batch phase, the glycerol fed batch at high glycerol uptake rates was carried out to maintain repressed conditions for biomass generation [1, 4, 14].

To familiarize cells to methanol, a methanol adaptation pulse of 0.5% (v/v) was performed. Subsequently the methanol induction fed batch was performed. As already observed in the

previous DASbox® cultivation, hypoxic conditions occurred again and therefore the absolute $q_{Protein}$ values still could not be trusted, nevertheless a comparison was feasible.

In Figure 10 the achieved $q_{Protein}$-rates were plotted against the controlled $q_{S,Methanol}$ steps (symbols: circle, square and triangle). Right from the first $q_{S,Methanol}$ step methanol accumulated for both promoter combinations $P_{DC}|P_{DF}$ and $P_{DF}|P_{GAP}$, which was indicated by flash-symbols, whereas methanol concentration for the $P_{DC}|P_{AOX1}$ promoter system started to rise in the course of the second $q_{S,Methanol}$-step. The delayed accumulation might presumably be attributed to the strong and methanol inducible P_{AOX1} promoter [4, 11, 15, 16]. A possible explanation for the fast methanol accumulation of the $P_{DC}|P_{DF}$ and $P_{DF}|P_{GAP}$ strains might be that the maximum methanol uptake rate of these two systems in contrast to $P_{DC}|P_{AOX1}$ was comparably lower. Whereas the methanol accumulation of the $P_{DC}|P_{AOX1}$ system happened right at $q_{S,max,MeOH}$, at the maximum methanol uptake limit. Although methanol was present in the cultivation broth, the concentrations in all three reactors never reached toxic conditions, since all strains produced further target protein. Studies using different *P. pastoris* strains expressing various recombinant proteins showed a negative impact on productivity only at methanol concentrations (c_{MeOH}) higher than c_{MeOH}= 10 [$g \cdot L^{-1}$] [17-19]. Based on the fact that the absolute methanol concentrations of each reactor never exceeded 0.6 [$g \cdot L^{-1}$], it can be assumed that no toxic conditions were existing and hampered the protein expression.

However, methanol accumulation over time (displayed as solid lines) also caused an increasing productivity in all three cases (compare Table 6). Considering the $q_{Protein}$ trends of each individual strain, it turned out that the *P. pastoris* strain carrying the $P_{DC}|P_{DF}$ promoter combination delivered the highest productivity. As can be seen, the promoter systems containing P_{DF} showed higher protein expression in both cases in contrast to the $P_{DC}|P_{AOX1}$ strain. This leads to the assumption that the modified *formaldehyde dehydrogenase* promoter is strongly induced by methanol, even more than the P_{DC} option.

Table 6: Summarized results of performed induction cultivation for the bi-directional promoter combinations $P_{DC}|P_{AOX1}$, $P_{DC}|P_{DF}$ and $P_{DF}|P_{GAP}$

$P_{DC}\|P_{AOX1}$		$P_{DC}\|P_{DF}$		$P_{DF}\|P_{GAP}$	
$q_{S,MeOH}$	$q_{Protein}$	$q_{S,MeOH}$	$q_{Protein}$	$q_{S,MeOH}$	$q_{Protein}$
$[g_S \cdot g_X^{-1} \cdot h^{-1}]$	$[mg_P \cdot g_X^{-1} \cdot h^{-1}]$	$[g_S \cdot g_X^{-1} \cdot h^{-1}]$	$[mg_P \cdot g_X^{-1} \cdot h^{-1}]$	$[g_S \cdot g_X^{-1} \cdot h^{-1}]$	$[mg_P \cdot g_X^{-1} \cdot h^{-1}]$
0.02	0.01	0.01	0.0	0.01	0
0.03	0.08	0.03	0.21	0.03	0.17
0.04	0.19	0.04	0.44	0.04	0.36

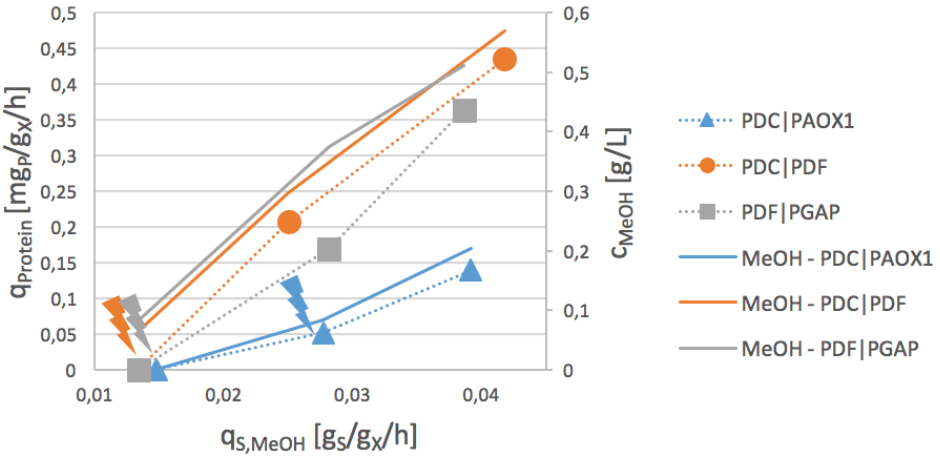


Figure 10: Results of second DASbox® cultivation summarized in a $q_{Protein}$ vs. $q_{S,Methanol}$ plot to see optimal operating window; flash symbol indicates start of methanol accumulation; solid lines indicates the increasing accumulated methanol concentration; circles, squares and triangles illustrate the increasing $q_{Protein}$ over. $q_{S,Methanol}$

Main outcome of Eppendorf DASbox® Cultivation

- The already found results of the promoter combination $P_{DC}|P_{AOX1}$ could be validated.
- It was possible to cultivate all novel *P. pastoris* strains. Furthermore, the strains were characterized successfully although hypoxic conditions cumbered the performed bioprocesses.
- The derepression glycerol fed batch showed, that the $P_{DF}|P_{GAP}$ strain delivered the highest productivity. This might explained by the constitutive P_{GAP} promoter, which shows strong protein production on carbon sources such as glycerol [13].
- Although, the promoter combinations $P_{DC}|P_{DF}$ and $P_{DF}|P_{GAP}$ already showed methanol accumulation right at the beginning, both resulted in higher protein production compared to the $P_{DC}|P_{AOX1}$ promoter system. However, the *P. pastoris* strain carrying the $P_{DC}|P_{DF}$ promoter system yielded in the highest $q_{Protein}$ rates. Due to the combination of two methanol inducible promoter options and assuming that P_{DF} is a very strong promoter, high protein expression could be achieved.

Based on the findings of the carried out experiments, it turned out that $P_{DF}|P_{GAP}$ is the system of choice when using a glycerol fed batch for highest productivity. On the contrary performing a methanol fed batch requires the use of the $P_{DC}|P_{DF}$ system for strong protein expression. However, too strong protein expression may not always be beneficial regarding correct protein folding [11]. For that reason, the apparently weaker $P_{DC}|P_{AOX1}$ system is a promising alternative. Depending on the desired results of the bioprocess, it is crucial to choose the most suitable bi-directional promoter combination.

Problems occured during this work

Balance Issues

Problem

During the first cultivation the process information management system (PIMS, Lucullus, Biospectra, Switzerland) suddenly recorded unreliable and oscillating values for the reactor weight as well as the substrate feed weight throughout the first $q_{S,Glycerol}$ controlled derepression step. The problem could be attributed to heavy vibrations caused by the increasing stirrer speed. The used bioprocess operation included the command to raise the speed of the stirrer in case of decreasing dissolved oxygen in the cultivation broth. Therefore, by reaching dO_2 concentrations lower than 30%, the agitation was regulated up and effected in an unstable and implausible balance signals.

However, the reactor weight as well as the feed weights were crucial for the subsequently performed data evaluation and strain characterization, it was key to immediately solve the problem.

Solution

Since we recognized rather strong vibrations in consequence of the high stirrer speed, which was needed for sufficient oxygen supply in the media, we steadied the reactor using tape to minimize movement (Figure 11). We did the same for the substrate feed flasks. In addition to that, a 2L shot flask bottle filled with water was placed beside the reactor to increase the total weight to even further reduce undesired vibrations which caused the oscillating weight values and complicated the data evaluation. The modified bioreactor setting was then used for all following cultivations to avoid inconvenient balance issues. Furthermore, to avoid any balance related complications, prior to each cultivation the successful recording of every balance was checked.

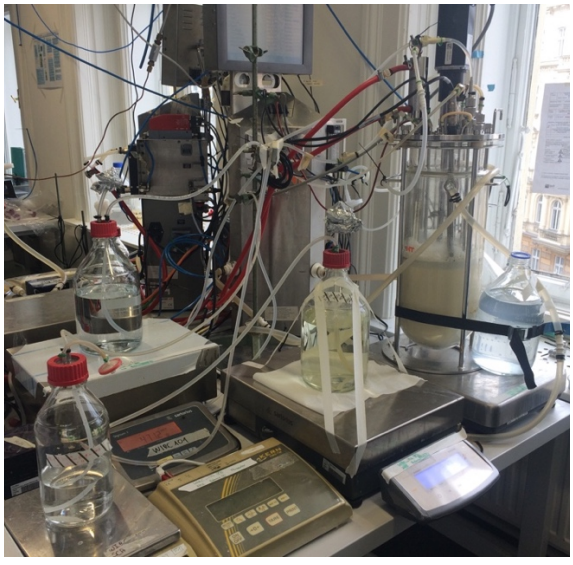


Figure 11: Modified bioreactor setting to reduce balance recording issues

“Siemens Box” Communication Issues

Problem

Different process parameters were monitored and controlled online by the process information management system “Lucullus” (PIMS, Biospectra, Switzerland). Figure 12 schematically illustrates the communication pathway between the individual parts of the bioprocess setting. During the second cultivation process, an electricity blackout resulted in a communication disruption between the bioreactor and the “Siemens Box” which is responsible for the command transmission of the management system to the bioreactor and its controllers.

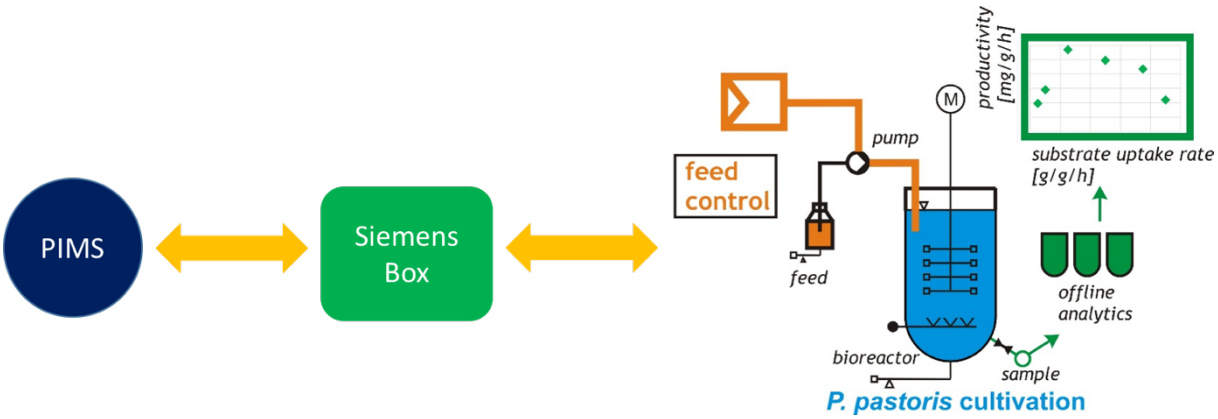


Figure 12: Simplified used bioprocess setting

Solution

It took a while to figure out the reason for the communication disruption. However, it appeared that the output signals of the “Siemens Box” for the substrate feed controllers were changed due to the electricity blackout. The issue was solved by reconfiguring the right output signal and the communication of all parts was restored.

Biomass determination for all bioprpcess cultivations

Problem

The cell dry weight biomass (CDW) determination was important for a correct followed data evaluation. Though, it turned out that the pipetting error of the plastic 5 mL micropipette resulted in CDW-value deviations greater than 5%.

Solution

The issue of great variations in CDWs could be solved by using a glass volumetric pipette instead of the 5 mL micropipette, which delivered stable and trustful CDW-biomass concentrations.

Hypoxic Conditions using the Eppendorf DASbox® Mini Bioreactor System

Problem

During the first as well as the second DASbox® cultivation the dissolved oxygen (dO_2) concentrations oscillated immensely causing stressful hypoxic conditions for the used cells. As already mentioned, oxygen limited conditions during a cultivation result in higher protein expression [7]. The higher productivity falsified the absolute values of the calculated total protein production rates. Since these obtained $q_{Protein}$ could not be compared to production rates delivered of the 2L scale cultivation, only the trends had been convinced.

Solution

The dO_2-concentrations were regulated and controlled by a PID-controller programmed by Dipl.-Ing. Julian Kager. For the second DASbox® cultivation the scripts of the PID-controller were adjusted based on previous findings. However, the oscillating could not be avoided entirely but at least it was possible to reduce them.

Missing second substrate feed in Eppendorf DASbox® Mini Bioreactor System

Problem

The second DASbox® cultivation required two separate substrate feeds to first generate biomass by applying glycerol and then inducing the promoters using a methanol feed. However, the used mini bioreactor system just provided a single substrate feed per reactor. Therefore, the setting could not be used for a concomitant mixed feed strategy.

Solution

Since two different feeds were required for the second cultivation, a special constructed two-way tubing system was developed to allow for first feeding glycerol for biomass growth and afterwards methanol for induction (see Figure 13). This could be achieved due to the complete closing of the methanol feeding tube using a metal clamp. By changing from glycerol to methanol feeding, the tubing coming from the glycerol reservoir was then closed by a metal clamp while the inlet of the methanol feed was opened. Due to the reason that the DASbox® Mini Bioreactor System did not use any feed balances for the evaluation of the amount of substrate that went into the reactor, the changing from glycerol to methanol feeding could be performed by closing and opening the respective tubes. The feeding rates of the pumps were monitored by the Eppendorf process information system. Based on calibration curves, the feed rates could afterwards be converted into weights and used for data evaluation. Hence, it was possible to use two different substrate feeds one after another.

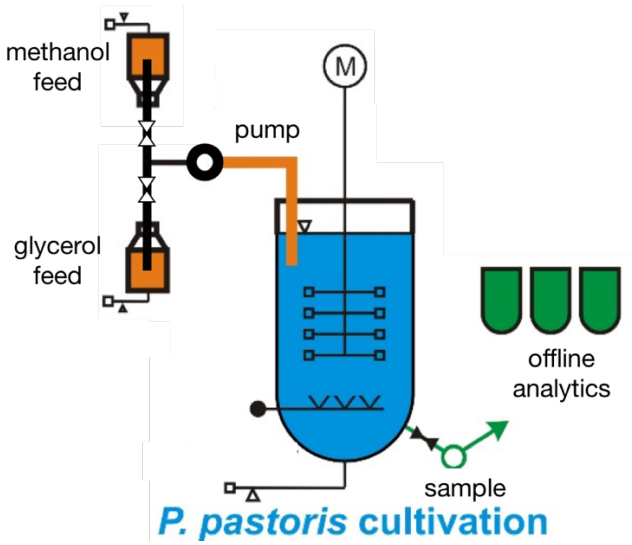


Figure 13: Schematic illustration constructed two way tubing system for feeding two substrates using one pump

Standard Operation Procedures (SOP)

All essential procedures which were carried out and standardized in course of this Master Thesis to obtain the prior shown results are summed up in this chapter.

SOP – Develop an Operation in Lucullus

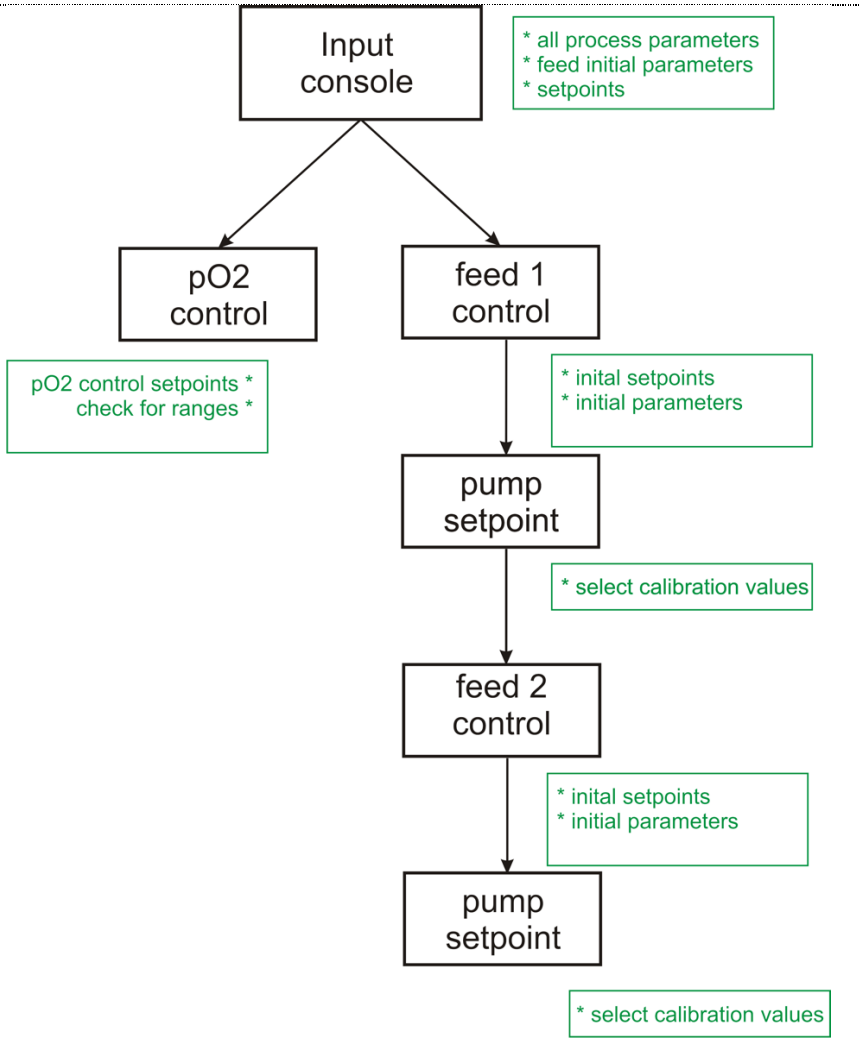
Standard Operation Procedure	**Designing Lucullus Operation**
Description	This SOP describes a method to develop and to write a bioprocess operation in the process information management system "Lucullus" (PIMS, Biospectra, Switzerland) for q_S-controlled fed batch cultivations.
Overall Workflow	

Figure 14: Schematic overall operation workflow for q_S-controlled fed batch phases in "Lucullus"

Equipment

- Computer connected to the TU Wien network able to access “Lucullus”

Procedure

- Open Lucullus and enter your login-data to get access to the control window (Figure 15):

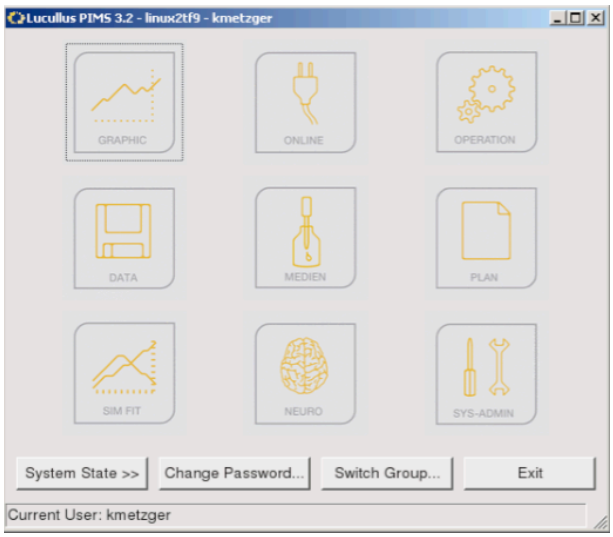


Figure 15: "Lucullus Control Window"

- To write new operations click on “Operations” to access the “Lucullus – Operation Tool” (Figure 16):

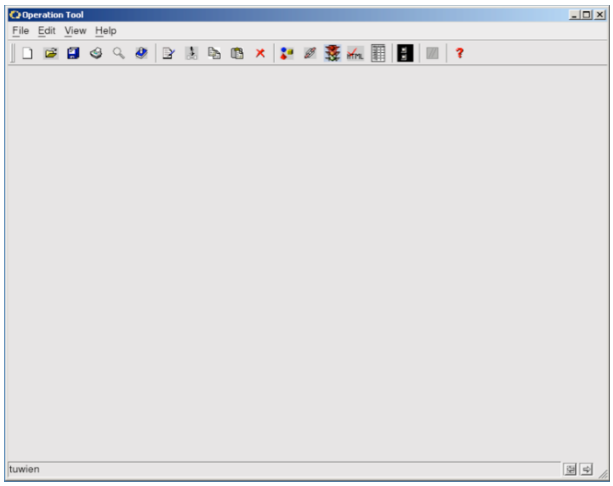


Figure 16: “Lucullus – Operation Tool”

- To create a new bioprocess operation first click on “File” and choose “New” – “New Operation”
- The “Resource Selection”- window (Figure 17) opens up where all required resources have to be selected such as the reactor and system devices (Calculator, Exponential Ramp, etc.) – The list of selected resources depends on

the planed cultivation plan. However, if a device is missing, it can be added at any moment just by switching to the “Resource Selection”- window (by clicking )

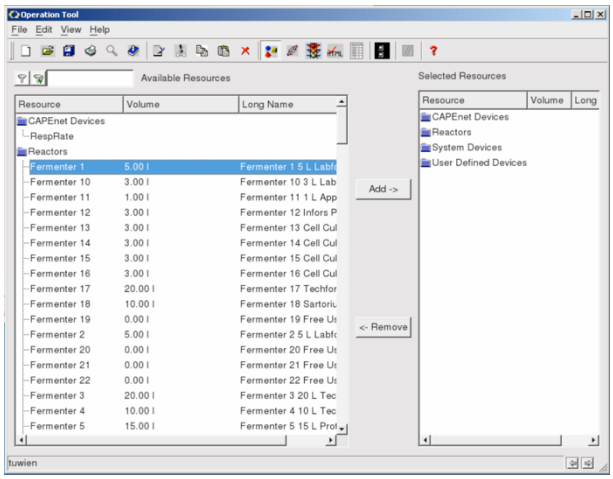


Figure 17: "Lucullus - resource selection window"

- The next step is needed to define all process parameters, initial feed parameters for fed batch cultivation, pO_2 control set points, various set points required for feed control and pump calibration values – Figure 14 schematically shows which parameter has to be defined in which operation step
- IMPORTANT: to develop a valid operation it is key to check the linkages between the defined parameters and the particular operation
- To verify the written operation click on  . If the operation is valid and can be used for bioprocess control a little window will pop up and say “Operation is valid” (Figure 18).

Figure 18: "Lucullus - operation is valid"

Formulas for Operation

- Initial feed rate for q_S-controlled bioprocesses:

$$FR_0 = \frac{c_X V_R}{c_S} q_{S,Glycerol}$$

FR_0 – initial feed rate [$L \cdot h^{-1}$]

c_X – calculated biomass wet weight concentration [$g_X \cdot L^{-1}$]

V_R – reactor volume [L]

$q_{S,Glycerol}$ – glycerol uptake rate [$g_S \cdot g_X^{-1} \cdot h^{-1}$]

c_S – glycerol feed concentration [$g_S \cdot L^{-1}$]

Used Lucullus Operation

This is the detailed "Lucullus"-Operation used for each bioprocess cultivation, including all commands and settings:

Batch-Fedbatch-Mixed_Feed-KME

Version 1

Reactors

Reactor	Scale [L]
Fermenter 1	5.0

Selected System Devices

Devices	Resources
Aeration Rate Calc	Calculator
Batch Initial Param	InputConsole
Calc_1_Gasmix	Calculator
Calc_2_Gasmix	Calculator
Fedbatch Initial Pa	InputConsole
Feedrate Calc MeOH	Calculator
Flow Controller Gly	Flow Controller
Flow Controller MeO	Flow Controller
FR1_at_t0	Calculator
FR2_at_t0	Calculator
PID Gly	PID Controller
PID MeOH	PID Controller
pO2_agitation	Step Controller
pO2_Gasmix	Step Controller
qS2_step_increase	Calculator
Setpoint Calc Gly	Calculator
Setpoint Calc MeOH	Calculator
System Device	System Device

Operation Steps

1. Shutdown

2. Begin – Initial Batch Parameters

Batch_Initial_Param__Input_01__LABEL = VL(t_batch) - Batch volume - L
Batch_Initial_Param__Input_01__INIT = 2
Batch_Initial_Param__Input_02__LABEL = pHw - pH Setpoint - pHU
Batch_Initial_Param__Input_02__INIT = 5
Batch_Initial_Param__Input_03__LABEL = pO2w - pO2 Setpoint - %
Batch_Initial_Param__Input_03__INIT = 30
pH_Start = On
pH_SptExt = Batch_Initial_Param__Input_02__VALUE
AgitatorSpeed_Start = On
ReactorTemperature_Start = On
ReactorTemperature_SptExt = 30
Batch_Initial_Param__Input_04__LABEL = vvm
Batch_Initial_Param__Input_04__INIT = 2
Batch_Initial_Param__StatusSubDevA__START = On
Batch_Initial_Param__Input_05__LABEL = Transition Condition = waiting
Batch_Initial_Param__Input_05__INIT = 10
Batch_Initial_Param__Input_06__LABEL = Transition Condition = Product Induction
Batch_Initial_Param__Input_06__INIT = 17
Batch_Initial_Param__Input_07__LABEL = Transition Condition = Induction1
Batch_Initial_Param__Input_07__INIT = 14
Batch_Initial_Param__Input_08__LABEL = Transition Condition = Split
Batch_Initial_Param__Input_08__INIT = 2
Batch_Initial_Param__Input_09__LABEL = Transition condiiton = Ready
Batch_Initial_Param__Input_09__INIT = 6

3. Fed Batch Initial Parameters – Glycerol

Fedbatch_Initial_Pa__Input_01__LABEL = c_XL (t_batchend) - Biomass - g/L
Fedbatch_Initial_Pa__Input_01__INIT = 40
Fedbatch_Initial_Pa__Input_02__LABEL = qS_max - Maxiumum Substrate Uptake Rate - g/g/h
Fedbatch_Initial_Pa__Input_02__INIT = 0.025
Fedbatch_Initial_Pa__Input_03__LABEL = V_L - Volume Bioreactor - L
Fedbatch_Initial_Pa__Input_03__INIT = 2
Fedbatch_Initial_Pa__Input_04__LABEL = c_S1R - Glycerol Conc in Reservoir - g/L
Fedbatch_Initial_Pa__Input_04__INIT = 300
Fedbatch_Initial_Pa__Input_12__LABEL = Pump Calibration Gly - Slope
Fedbatch_Initial_Pa__Input_12__INIT = 0.0037
Fedbatch_Initial_Pa__Input_14__LABEL = Intercept - Gly Pump Calibration
Fedbatch_Initial_Pa__Input_14__INIT = 0.0019
Fedbatch_Initial_Pa__StatusSubDevA__START = On
Fedbatch_Initial_Pa__StatusSubDevA__INTERVAL = 10
Fedbatch_Initial_Pa__Input_16__LABEL = M_X - Molecular Weight Biomass - g/C-mol
Fedbatch_Initial_Pa__Input_16__INIT = 25.74
Fedbatch_Initial_Pa__Input_17__LABEL = M_S1 - Molecular Weight Glycerol - g/C-mol
Fedbatch_Initial_Pa__Input_17__INIT = 36

4. Fed Batch Initial Parameters – Methanol

Fedbatch_Initial_Pa__Input_06__LABEL = Maximum Substrate Uptake rate - qSMeOH - g/g/h
Fedbatch_Initial_Pa__Input_06__INIT = 0.03
Fedbatch_Initial_Pa__Input_08__LABEL = c_S2R - Methanol Conc. in Reserv. - g/L
Fedbatch_Initial_Pa__Input_08__INIT = 250
Fedbatch_Initial_Pa__Input_09__LABEL = Pump Calib. slope - Methanol
Fedbatch_Initial_Pa__Input_09__INIT = 0.0196
Fedbatch_Initial_Pa__Input_10__LABEL = Pump calib. intercept - Methanol
Fedbatch_Initial_Pa__Input_10__INIT = -0.0189
Fedbatch_Initial_Pa__Input_11__LABEL = M_S2 - Molecular Weight MeOH - g/C-mol
Fedbatch_Initial_Pa__Input_11__INIT = 32.04

5. Aeration Rate Calculator

Aeration_Rate_Calc__CalculatorSubDev__S1 = Batch_Initial_Param__Input_01__VALUE
Aeration_Rate_Calc__CalculatorSubDev__S2 = Batch_Initial_Param__Input_04__VALUE
*Aeration_Rate_Calc__CalculatorSubDev__Formula = S1*S2*
Aeration_Rate_Calc__StatusSubDevA__START = On
AIRIN_Start = On
AIRIN_SptExt = Aeration_Rate_Calc__CalculatorSubDev__Result

6. 1 Begin

Go to “7. pO_2 Control Agitation” and “14. Batch”

7. pO_2 Control Agitation

pO2_agitation__ProcValSubDev__PVCur = DO2Redox
pO2_agitation__ProcValSubDev__PVHigh = 150
pO2_agitation__ProcValSubDev__PVLow = Batch_Initial_Param__Input_03__VALUE
pO2_agitation__SetPointSubDev__YHigh = 1210
pO2_agitation__SetPointSubDev__YInc = -10
pO2_agitation__SetPointSubDev__YLow = 1100
pO2_agitation__SetPointSubDev__YStart = 600
pO2_agitation__StatusSubDevA__START = On
pO2_agitation__StepCtrlSubDev__StepPeriod = 30
AgitatorSpeed_Start = On
AgitatorSpeed_SptExt = pO2_agitation__SetPointSubDev__YCur
pO2_agitation__StatusSubDevA__INTERVAL = 1

If Agitator Speed>1200, then go to “8. pO_2 Control Gasmix”

8. pO_2 Control Gasmix

pO2_Gasmix__ProcValSubDev__PVCur = DO2Redox
pO2_Gasmix__ProcValSubDev__PVHigh = 150
pO2_Gasmix__ProcValSubDev__PVLow = Batch_Initial_Param__Input_03__VALUE
pO2_Gasmix__SetPointSubDev__YHigh = 0.75
pO2_Gasmix__SetPointSubDev__YInc = -0.001
pO2_Gasmix__SetPointSubDev__YLow = 0
pO2_Gasmix__SetPointSubDev__YStart = 0
pO2_Gasmix__StepCtrlSubDev__StepPeriod = 10
Calc_1_Gasmix__CalculatorSubDev__S1 = Aeration_Rate_Calc__CalculatorSubDev__Result
Calc_1_Gasmix__CalculatorSubDev__S2 = pO2_Gasmix__SetPointSubDev__YCur
*Calc_1_Gasmix__CalculatorSubDev__Formula = S1*S2*
Calc_2_Gasmix__CalculatorSubDev__S1 = Aeration_Rate_Calc__CalculatorSubDev__Result
Calc_2_Gasmix__CalculatorSubDev__S2 = Calc_1_Gasmix__CalculatorSubDev__Result
Calc_2_Gasmix__CalculatorSubDev__Formula = S1-S2
OxygenIN_SptExt = Calc_1_Gasmix__CalculatorSubDev__Result
AIRIN_SptExt = Calc_2_Gasmix__CalculatorSubDev__Result
AIRIN_Start = On
O2Line_Start = On
Calc_1_Gasmix__StatusSubDevA__START = On
Calc_2_Gasmix__StatusSubDevA__START = On
pO2_Gasmix__StatusSubDevA__START = On
pO2_Gasmix__StatusSubDevA__INTERVAL = 1

9. Initial Feed Rate – Glycerol Fed Batch

FR1_at_t0__CalculatorSubDev__S1 = Fedbatch_Initial_Pa__Input_01__VALUE
FR1_at_t0__CalculatorSubDev__S2 = Fedbatch_Initial_Pa__Input_02__VALUE
FR1_at_t0__CalculatorSubDev__S3 = Fedbatch_Initial_Pa__Input_03__VALUE
FR1_at_t0__CalculatorSubDev__S4 = Fedbatch_Initial_Pa__Input_04__VALUE
*FR1_at_t0__CalculatorSubDev__Formula = (S1*S2*S3*S7)/(S4*S8)*
FR1_at_t0__StatusSubDevA__START = On
FR1_at_t0__StatusSubDevA__INTERVAL = 10
FR1_at_t0__CalculatorSubDev__S7 = Fedbatch_Initial_Pa__Input_17__VALUE
FR1_at_t0__CalculatorSubDev__S8 = Fedbatch_Initial_Pa__Input_16__VALUE

If System_Device__SystemSubDev__Phase = 6, then go to “10. Setpoint for Glycerol Pump”

10. Setpoint for Glycerol Pump

Setpoint_Calc_Gly__CalculatorSubDev__S1 = Fedbatch_Initial_Pa__Input_12__VALUE
Setpoint_Calc_Gly__CalculatorSubDev__S2 = FR1_at_t0__CalculatorSubDev__Result
Setpoint_Calc_Gly__CalculatorSubDev__S3 = Fedbatch_Initial_Pa__Input_14__VALUE
Setpoint_Calc_Gly__StatusSubDevA__INTERVAL = 10
Setpoint_Calc_Gly__StatusSubDevA__START = On
Setpoint_Calc_Gly__CalculatorSubDev__Formula = (S2-S3)/S1
Feed2_Start = On
Feed2_SptExt = Setpoint_Calc_Gly__CalculatorSubDev__Result

If System_Device__SystemSubDev__Phase = 17, then go to “16. Mixed Feed Start End”

If System_Device__SystemSubDev__Phase = 14, then go to “9. Initial Feed Rate – Glycerol Fed Batch”

11. Initial Feed Rate – Methanol Fed Batch

FR2_at_t0__CalculatorSubDev__S1 = Fedbatch_Initial_Pa__Input_01__VALUE
FR2_at_t0__CalculatorSubDev__S2 = Fedbatch_Initial_Pa__Input_06__VALUE
FR2_at_t0__CalculatorSubDev__S3 = Fedbatch_Initial_Pa__Input_03__VALUE
FR2_at_t0__CalculatorSubDev__S4 = Fedbatch_Initial_Pa__Input_08__VALUE
*FR2_at_t0__CalculatorSubDev__Formula = (S1*S2*S3*S7)/(S4*S8)*
FR2_at_t0__StatusSubDevA__START = On
FR2_at_t0__StatusSubDevA__INTERVAL = 10
FR2_at_t0__CalculatorSubDev__S7 = Fedbatch_Initial_Pa__Input_11__VALUE
FR2_at_t0__CalculatorSubDev__S8 = Fedbatch_Initial_Pa__Input_16__VALUE

If System_Device__SystemSubDev__Phase = 6, then go to “12. Setpoint for Methanol Pump”

12. Setpoint for Methanol Pump

Setpoint_Calc_MeOH__CalculatorSubDev__S1 = Fedbatch_Initial_Pa__Input_09__VALUE
Setpoint_Calc_MeOH__CalculatorSubDev__S2 = FR2_at_t0__CalculatorSubDev__Result
Setpoint_Calc_MeOH__CalculatorSubDev__S3 = Fedbatch_Initial_Pa__Input_10__VALUE
Setpoint_Calc_MeOH__StatusSubDevA__INTERVAL = 10
Setpoint_Calc_MeOH__StatusSubDevA__START = 1
Setpoint_Calc_MeOH__CalculatorSubDev__Formula = (S2-S3)/S1
Feed3_SptExt = Setpoint_Calc_MeOH__CalculatorSubDev__Result
Feed3_Start = On

If System_Device__SystemSubDev__Phase = 14, then go to “11. Initial Feed Rate – Methanol Fed Batch”

If System_Device__SystemSubDev__Phase = 17, then go to “16. Mixed Feed Start End”

13. Begin End

Shutdown

14. Batch

If System_Device__SystemSubDev__Phase = 2, then go to “15. Mixed Feed Start”

15. Mixed Feed Start

Go to “9. Initial Feed Rate – Glycerol Fed Batch” and “11. Initial Feed Rate – Methanol Fed Batch”

16. Mixed Feed Start End

Go to “13. Begin End”

Standard Operation Procedure	Determination of Cell Dry Weight
Description	This SOP describes a method to determine the biomass cell dry weight (CDW) concentration gravimetrically. The CDW-concentrations can then be used for further data evaluation required for strain characterization and determination of particular physiological strain parameters. For trustful data at least triplicates need to be done.
Materials	• 10 mL glass tube • 5 mL volumetric pipette and BRAND® macro pipette controller • Distilled water
Equipment	• Analytical balance connected to computer for recording (ME 204; Mettler Toledo, Switzerland) • Incubator chamber at 95°C (Heraeus; Type: ST 5050) • Temperature-controlled centrifuge at 4°C (Sigma 3-18K; Rotor Sigma 11133) • Vortex (VORTEX-GENIE 2; Scientific Industries) • Glass desiccator containing silicagel
Procedure	• Glass tubes (W_{Empty}) must be weighted, cleaned and room-temperated using the analytical balance (important: glass tube must be placed at the very center of the balance platform) • Using the volumetric pipette 5 mL, each 5 mL of culture broth is transferred to the pre-weighted glass tubes • The filled tubes are immediately centrifuged at 4°C at 4800 rpm (approx. 4000 g) for 10 minutes • Aliquots of the supernatants may be used for HPLC analysis if needed. Otherwise they will be discarded.

- 5 mL of distilled water is added to the biomass pellets and resuspended by the help of a Vortex for washing
- The washed cells are centrifuged again at 4°C at 4800 rpm (approx. 4000 g) for 10 minutes
- The supernatants are discarded (carefully)
- The tubes containing the biomass are then placed in the incubation chamber at 90°C and dried for 72 hours
- After 72 hours the tubes are placed in the glass desiccator for cooling down to room temperature
- The filled, room-temperated tubes are weighted using the analytical balance (W_{Full})

Calculations

- The cell dry weight concentration can be calculated using following formula:

$$CDW\ \left[\frac{g}{L}\right] = \left(W_{Full} - W_{Empty}\right) * 200$$

Standard Operation Procedure	Determination of Optical Density at 600 nm and Dry Cell Weight Concentration
Description	This SOP describes a method to determine the optical density at 600 nm and further the calculation of dry cell weight (DCW) concentration. The optical density method can therefore be used for almost real-time DCW-concentration determination required e.g. for controlling a feed forward cultivation.
Materials	• 1 mL plastic cuvettes • 1 mL micropipette and proper tips • 200 µL micropipette and proper tips • Distilled water
Equipment	• Lab photometer (Thermo Scientific, GENESYS20)
Procedure	• Before the measuring can be performed the photometer must run at least 10 min • Choose the mode ABS (Absorbance) for optical density measurement • The wavelength has to be adjusted to 600 nm by using the buttons “UP” and “DOWN” • Prior to the actual OD-measurement a blank with 1 mL distilled water in a plastic cuvette has to be prepared • The blank cuvette needs to be placed in the right direction (small arrow indicates the light beam) inside the photometer and the lid has to be closed • Press the button “0 ABS 100% T” when the value shown on the display is stable – the system is blanked and the blank cuvette can be removed (important: the lid must be closed all the time) • Vortex the cultivation broth sample and pipette 1 mL of the well mixed sample into a plastic cuvette and vortex it

- Place the sample inside the photometer in the right direction and close the lid
- Note the measured value (ABS)
- If the value exceeds 1.0 ABS, the cultivation broth sample has to be diluted with water appropriately since the linear range of the photometer serves from 0.1 to 1.0 ABS.

- The optical density at 600 nm can be calculated using following formula. The dilution factor has to be taken in account if the samples are diluted for measuring.

$$OD_{600} = ABS * Dilution\ Factor$$

Calculations

- The dry cell weight concentration (DCW) can be calculated using following formula. The empirical determined α-factor describes the correlation between OD_{600} value and dry cell weight (DCW) of previous cultivations.

$$DCW\ \left[\frac{g}{L}\right] = OD_{600} * \alpha$$

Standard Operation Procedure	Determination of Total Protein Content
Description	This SOP describes a method to determine the total protein content using the commercial available Bradford Reagent. This photometrical method is based on the chemical reaction between proteins and the Brilliant Blue G-dye.
Materials	• 1 mL plastic cuvettes • 1 mL micropipette and proper tips • 200 µL micropipette and proper tips • 20 µL micropipette and proper tips • Bradford Reagent (Sigma-Aldrich; Cat. No.: B6916) • Distilled water
Equipment	• Lab photometer (Thermo Scientific, GENESYS20)
Procedure	• Before the measuring can be performed the photometer must run at least 10 min • Choose the mode ABS (Absorbance) for optical density measurement • The wavelength has to be adjusted to 595 nm by using the buttons “UP” and “DOWN” **Blank/Sample preparation** • Prefill empty plastic cuvettes with Bradford reagent. The amount of reagent depends on the desired final dilution – e.g. if low protein concentrations are expected a 1:10 dilution might be a good option (990 µL Bradford reagent + 10 µL sample) • Then the relevant volume of sample has to be added to the Bradford reagent and mixed well using a Vortex • A blank has also to be prepared. The blank consist of distilled water/buffer with no protein

- The samples as well as the blank are incubated at room temperature for 15 minutes by exclusion of light

Measurement

- After 15 minutes the samples are ready to be measured
- First the blank cuvette will be vortexed and then placed in the right direction (small arrow indicates the light beam) inside the photometer. Close the lid
- Press the button “0 ABS 100% T” when the value shown on the display is stable – the system is blanked and the blank cuvette can be removed (important: the lid must be closed all the time)
- Vortex each sample before measuring
- Place the sample inside the photometer in the right direction and close the lid
- Note the measured value (ABS)
- If the value exceeds 1.0 ABS, the samples either has to be diluted with water appropriately or another Bradford reagent-sample dilution need to be prepared since the linear range of the photometer serves from 0.1 to 1.0 ABS.

Calculations

- Prior to the calculation of the actual protein concentration of the measured sample, bovine serum albumin protein standard with known concentrations has to be measured exact the same way as the sample
- The measured protein standard absorption values are plotted against the corresponding concentrations to construct a regression model
- The model is then used to calculate the actual concentration of the unknown samples

Standard Operation Procedure	Determination of Substrate Concentrations via HPLC
Description	This SOP describes a method to determine substrate concentrations in the supernatant of fermentation broth samples using high pressure liquid chromatography techniques (HPLC). The method relies on the differing retention times of various secondary metabolites using an ion-exchange column.
Materials	• 0.2 µm sterile filter • 1 mL micropipette and proper tips • HPLC glass vials • (for small sample volume: 50 µL glass vial insert) • MilliQ water • 85% H_3PO_4 • NaN_3 • Standard serial dilution set of substrate of interest
Equipment	• Dionex UltiMate 3000 HPLC (Thermo Scientific, USA) • SUPELCOGEL™ C-610H HPLC column (Sigma-Aldrich, Cat. No.: 59320-U) • RI-Detector RI-101 (Shodex)
Procedure	**Mobile Phase Preparation** • Dilute phosphoric acid to a final concentration of 0.1% using MilliQ water • One spatula tip of NaN_3 is added • Place the solution inside an ultrasonic-bath for degassing • The degased mobile phase is then transferred into the correct solvent bottle of the HPLC **Sample Preparation**

- All samples need to be sterile filtered using 0.2 µm filter
- At least 250 µL of each sample has to be pipetted into a HPLC glass vial each (if sample volume is less than 250 µL, use a 50 µL glass vial insert)
- Prepare one HPLC vial with 1 mL MilliQ water
- Place the samples, the MilliQ sample as well as the standard serial dilution set in the correct order in the HPLC auto sampler tray

HPLC-Operation Procedure

- To start the operation, the HPLC as well as the connected computer is switched on
- Then the corresponding software is opened
- Afterwards the sequence Table of all samples is written, including sample order, sample name, sample injection volume and desired HPLC method

  HPLC method settings:
  - Flow-Rate: 0.5 [mL/min]
  - Sample injection volume: 10 [µL]
  - Run time: 30 [min]
  - Temperature: 30 [°C]
- Before starting the HPLC measurement the whole system has to be purged for at least 15 minutes
- IMPORTANT: insert the actual volume of mobile phase in the storage tank and enter the exact value into the software
- The Sequence is started – wait untill the first sample is loaded to see if the measurement in fact has been started

Data evaluation

- The obtained chromatograms can then be further processed by integrating the measured peaks using the corresponding integration software

- By plotting the determined peak areas of the standards against the corresponding concentrations a regression model can be constructed and used for calculating the unknown sample concentrations

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