BI-VARIATE GROWTH MODEL OF *PICHIA PASTORIS* INCLUDING OXYGEN CONSIDERATIONS AND ITS IMPORTANCE IN RECOMBINANT PROTEIN PRODUCTION

by

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To all those who chase their passions,
no matter when they call or where they lead

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ABSTRACT

Author: Binkley, Robert, M. MSABE
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Title: Bi-Variate Growth Model of *Pichia Pastoris* including Oxygen Considerations and its Importance in Recombinant Protein Production
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The methylotrophic yeast, *Pichia pastoris* (recently reclassified as *spp. Komagatella*) has long been regarded as a useful host organism for the production of recombinant proteins, particularly when using the AOX system which utilizes methanol as both the inducing agent as well as the primary carbon source for growth and energy. Significant historical work has shown that growth rate and protein productivity can be correlated to methanol concentration. However, the relationship between oxygen and protein productivity are less consistent. While with many variations models having been developed and used for analyzing culture kinetics, these models have only been applied to methanol concentration. Furthermore, while results for methanol are fairly consistent, oxygen considerations have been far less consistent.

This work presents various bi-variate models which includes considerations for growth and inhibition for both methanol and oxygen with this expanded model showing strong alignment to previous works to both oxygen and methanol data. While more work is necessary to fully confirm and validate which form of the bivariate model is most appropriate, this work provides a framework necessary to expand analysis to include oxygen considerations. This framework has the potential to be used to further inform selection of feeding methodology as well as direct investigations into metabolic studies.

CHAPTER 1: INTRODUCTION

The microbial conversion of chemicals, a term more broadly termed as fermentation, is one of the oldest technologies utilized by humans. Applications of applied protein fermentations can date back to ~500BC, where Chinese texts describe the application of mold (*Aspergillus oryzae*) in the product of rice wine; these molds produced a mixture of enzymes and amylases required for rice fermentation. Microbial protein production can trace its roots back to birth of modern biochemistry, where Louis Pasteur (1822-1895) and Eduard Buchner (1860-1917) used cell free extracts of yeast in order to study the effects of fermentation, with the extracts serving as crude protein mixtures. One of the earliest applications of submerged bacterial fermentations for the production of proteins was that of Auguste Boidin and Jean Effront, (US Patent 1, 227, 374) who grew cultures of *Bacillus subtilis* and *B. mesentericus* for the production of amylase. Microbial production of recombinant proteins has become even more significant, with microbes being used industrially to produce a wide array of proteins, from pharmaceuticals, such as antibodies, growth factors, and interferons/interleukins, to industrially enzymes [Lakowitz 2018, Safder 2018].

The production of rProteins in a commercially relevant fashion often involves production in large scale reactors, and therefore scale up considerations are imperative to their economic viability. Fed-batch processes are often used for fermentations when compared to batch processes for a multitude of reasons, including; A) they often allow for increased concentration of final products due additional substrate being added and B) they allow for precise control of operational factors, such as pH or substrate concentration, since additional material can be added which affect these parameters. For microbial fed-batch fermentations, the feed is often a concentrated carbon source (typically a sugar) and may contain other trace nutrients or other defined chemicals. The method in which this feed is added to the process however can have a significant impact on culture performance, from production formation rates, to yield and byproduct formation, to biomass formation. Since microbial production of proteins, especially recombinant proteins, is not directly connected with natural (inherent) metabolic functions, like ethanol and lactic acid, production can range over orders of magnitude, from the µg/L to 100 g/L, making it is difficult to predict and model production kinetics. Even if it is difficult to predict for a specific protein, considerations on

growth in general can be valuable tools when directing experiments effectively to lead to a final product

Having long been regarded as one of the most important cell factories for the production of recombinant proteins and bio-pharmaceutical products, the yeast *Pichia pastoris* (*Komagataella spp*) has been used in the production of various proteins, from antibody fragments for syncytial virus (RSV) infection treatment to recombinant proteins for animal feed nutrient additives [Safder, 2018; Pichia.com, 2018]. While many promoters and expression systems have been developed, the *AOX1* gene and PAOX1 promoter (generally called "the AOX system") is widely used for recombinant protein production due to tight regulation, strong induction by methanol, and historically high protein production [Potvin 2012, Emenike 2018]. Since achieving maximum productivity is paramount to developing economic and cost effective processes, substantial effort has been made in order to optimize fed-batch production, from development of various feeding methods [Looser 2015] to utilizing mixed substrate feeds [Cos 2006].

CHAPTER 2: GENERAL PRODUCTION OF RECOMBINANT PROTEINS IN YEASTS

With the advances in molecular biology tools, microbial production of targeted recombinant proteins is possible in many different species, from bacteria to insect and mammalian cultures. While each system has their advantages and disadvantages, yeast systems, being eukaryotes, allow for more complex protein formation than prokaryotes (i.e. bacterial systems) while also generally having faster growth rates than mammalian systems. Furthermore, resistance of yeast to shear due to beta-glucans in the cell membrane, can allow for increased agitation rates (and subsequently high oxygen transfer) while still maintaining cell viability.

Specifically, this work will focus on the yeast *Pichia pastoris*; however, in order to help motivate the context of rProtein expression in *Pichia*, it is important to identify unique features of *Pichia* within yeast as a whole. One method of differentiating between various yeast species is categorizing them based on their metabolisms, particularly the metabolic pathways associated with glucose assimilation and overflow metabolism; this overall process is more commonly known as the Crabtree effect. Crabtree positive yeast are often characterized by high glucose uptake rates and metabolic flux of the central carbon metabolism toward the production and secretion of ethanol [Christen 2011]. Heterologous protein production has been performed in both Crabtree negative and positive yeasts (Table 1). However, in terms their potential to produce protein products, Crabtree negative yeasts are thought to have an inherent advantage over Crabtree positive species: due to their lack of overflow metabolism, there is less opportunity for carbon to be directed towards chemical byproducts and metabolites (such as ethanol) so that carbon flux is conserved in the TCA cycle for energy production (Figure 1). While this results in generally faster growth rates for Crabtree negative species [Christen 2011] it requires the cell be maintained in an aerobic environment (i.e. oxygen must be available). *Pichia pastoris* falls into this second group and is considered to be Crabtree negative [Porro 2005, Heyland 2011].

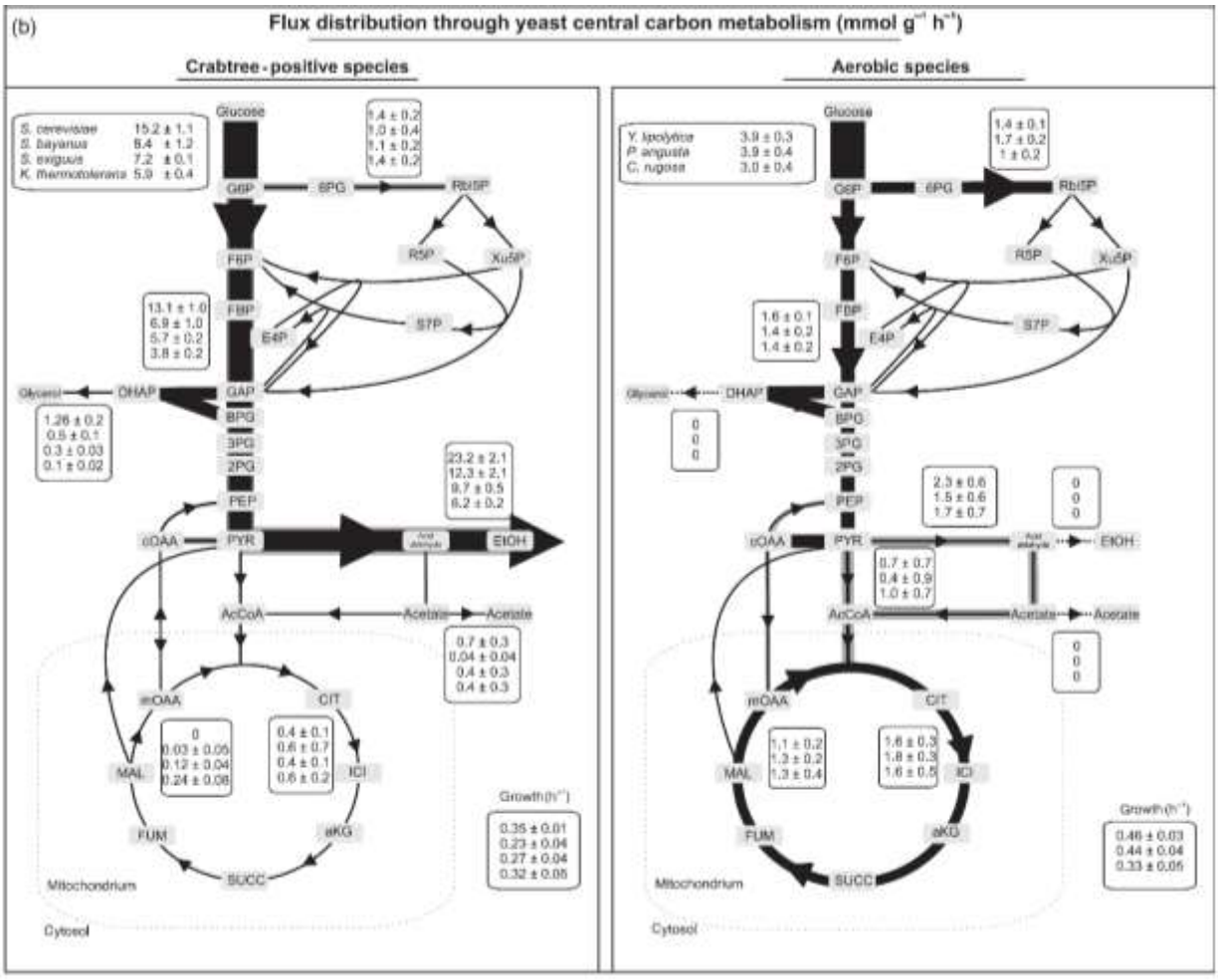

Figure 1: Quantitative flux distribution in seven yeast species (Crabtree-postitive (left) and aerobic (right)) grown on glucose; thickness of the arrows indicates glucose uptake normalized flux distribution; from [Christen 2011]

Table 1: List of recombinant proteins produced in various Crabtree negative and Crabtree postitive yeast systems

Crabtree Status	Organism	Protein	Expression level	Citation
Crabtree Positive	*Saccharomyces cerevisiae*			
		Human serum albumin	150 mg/L	Sleep 1991
		Tetanus toxin fragment C	1 g/L	Romanos 1991
		Glucose oxidase	9 g/L	Park 2000
		Hirudin	460 mg/L	Sohn 1995
		Human proinsulin	1.5 g/L	Tøttrup 1990
	Schizosaccharomyces pombe			
		Neurokinin NK2	1.16 pmol/mg	Arkinstall 1995
		D2S-Dopaminergic	14 pmol/mg	Sander 1994
		D2-Dopaminergic	1 pmol/mg	Presland 1998
		Antithrombin III	85 mg/L	Broker 1987
Borderline Crabtree	*Kluyveromyces lactis*			
		ovine β-lactoglobulin	40-50 mg/L (supernatant)	Rocha 1996
		Human Serum Albumin	3 g/L	Fleer 1991
Crabtree Negative	*Pichia pastoris*			
		Synthetic Insulin Precursor (codon optimized)	3.075 g/L total; 3.84 supernatant	Gurramkonda 2010
		HBsAg	7 g/L total; 2.3 g/L soluble	Gurramkkonda 2009
		Porcine Insuline Precursor	0.9 g/L	Chen 2017
		Lipase	3.3 g/L	Wang 2012
		Fab fragment	2.116 g/L	Buchetics 2011
		Tetanus toxin fragment C	12 g/L	Clare 1991
		Mab	1.6 g/L	Ye 2011
	Ogataea polymorpha			
		Phytase	13.5 g/L	Mayer 1999
		Hirudin	>1 g/L	Weydemann 1995

Pichai pastoris is also classified as a "methylotrophic yeast", in that it has native metabolic pathways necessary for utilizing methanol as a carbon source for biomass production and energy synthesis [Hartner 2006]. This methanol utilization (MUT) pathway is tightly regulated at the transcription level. The enzymes required for the first step of the MUT pathway (alcohol oxidases, EC 1.1.3.13) are controlled by the AOX1 and AOX2 promoter [Cereghino 2000] which has been successfully utilized as an inducible promoter for the production of recombinant proteins [Ahmad 2014].

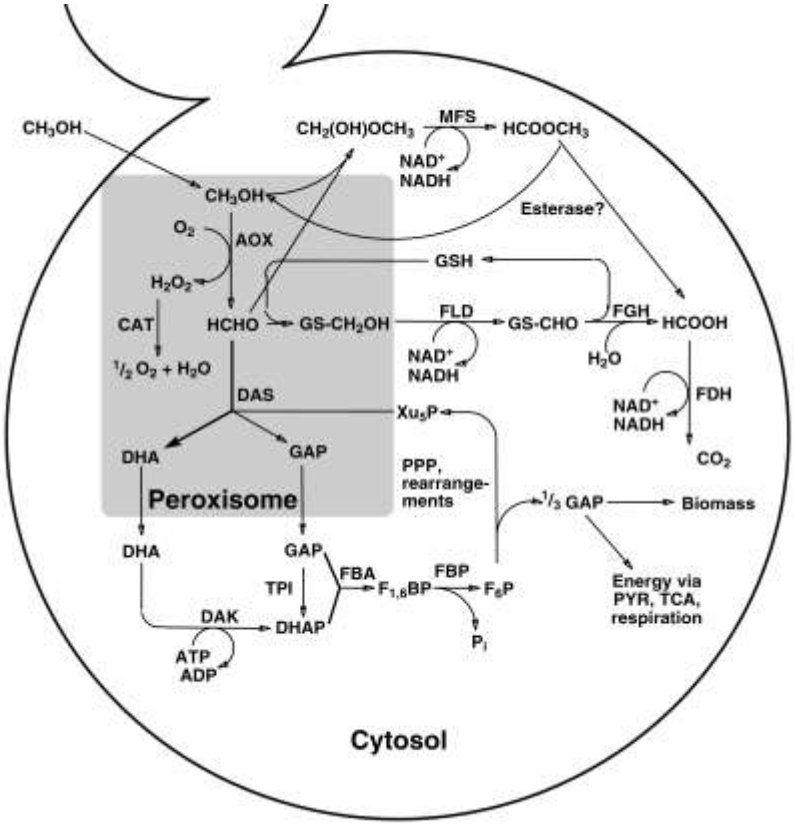

Figure 2: Methanol utilization pathway in methylotrophic yeasts; AOX: alcohol oxidase (EC 1.1.3.13), CAT: catalase (EC 1.11.1.6), FLD: formaldehyde dehydrogenase (EC 1.2.1.1), FGH: S-formylglutathione hydrolase (EC 3.1.2.12), FDH: formate dehydrogenase (EC 1.2.1.2), DAS: dihydroxyacetone synthase (EC 2.2.1.3), TPI: triosephosphate isomerase (EC 5.3.1.1), DAK: dihydroxyacetone kinase (EC 2.7.1.29), FBA: fructose 1,6-bisphosphate aldolase (EC 4.1.21.13), FBP: fructose 1,6-bisphosphatase (EC 3.1.3.11), MFS: methylformate synthase; DHA: dihydroxyacetone, GAP: glyceraldehyde 3-phosphate, DHAP: dihydroxyacetone phosphate F1,6BP: fructose 1,6-bisphosphate, F6P: fructose 6-phosphate, Pi: phosphate, Xu5P: xylulose 5-phosphate, GSH: glutathione, PYR: pyruvate; PPP: pentose phosphate pathway, TCA tricarboxylic acid cycle from [Hartner 2006]

It is interesting to note that the chemical intermediates of the methanol assimilation pathway include hydrogen peroxide as well as formaldehyde, two highly reactive chemical species which are known to be toxic for microbes; due to their highly reactive nature, these highly reactive chemical, as well as the enzymes that interact with them are generally contained in the peroxisome, which can account for a significant portion of the cellular volume during growth on methanol [Rußmayer 2015].

Due to its significance as a protein producing system, significant work has been performed looking at the effects of methanol concentration on the growth and protein producing capabilities of *Pichia*. Consistaently, methanol concentration has been shown to affect culture growth rate, which has led to the development of unstructured growth models used for experimental analysis [Zhang 2000, Barrigon 2015]. While exact values vary, growth increases steadily as methanol concentration increases, with maximum grow rate typically occurring at a critical methanol substrate concentration between 2-5 g/L, after which growth rate decreases at higher methanol concentrations. This type of growth rate dependence on methanol is conserved across different expressed protein targets (Figure 3). Recent works have confirmed the assumption that methanol transport is diffusion limited, giving further acceptance to methanol concentration dependent growth of *Pichia* [Singh 2019].

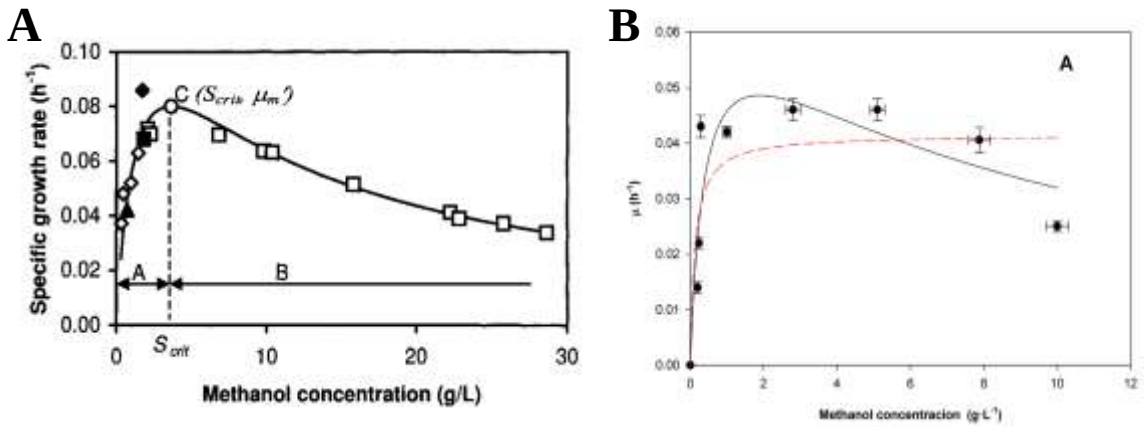

Figure 3: Methanol concentration dependence of growth rate in *Pichia pastoris*; A: unstructured growth model for *P. pastoris* expressing the heavy-chain fragment C of botulinum serotoxin serotype A [BoNT/H(Hc)] from [Zhang 2000], B: unstructured macrokinetic model for *P. pastoris* producing a *Rhizopus oryzae* lipase (ROL) from [Barrigon 2015]; points represent experimental data while lines represent model curves.

Furthermore, growth rate has been shown to correlate to protein productivity in *Pichia*, especially for protein production utilizing the AOX promoter and methanol. However, for AOX based systems, highest protein productivity is typically found at a growth rate well below the maximum (Figure 4).

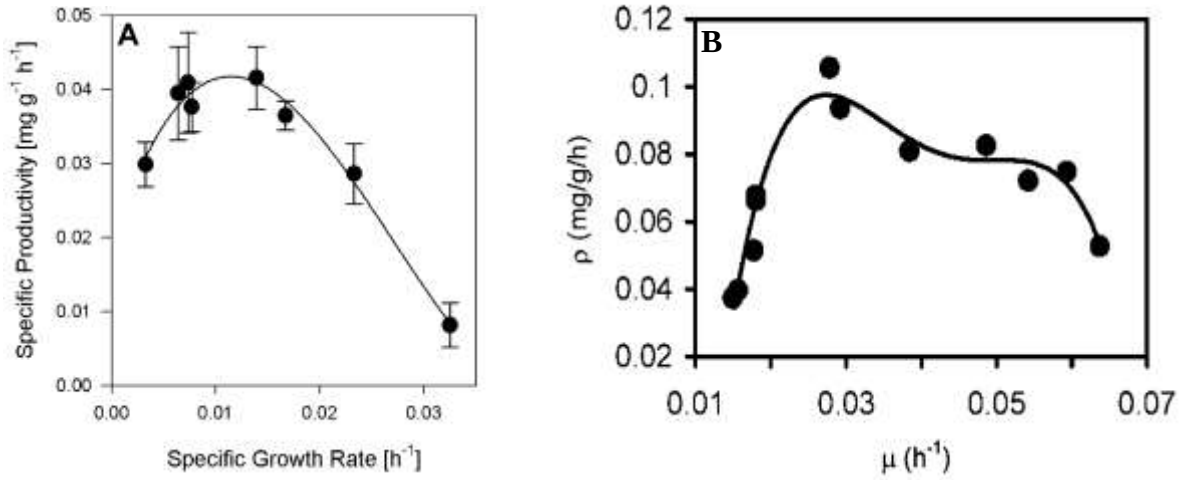

Figure 4: Growth rate dependence of protein productivity in *Pichia pastoris;* A: specific protein productivity for *Pichia* an IgG1 heavy and light chain from [Potgieter 2010], B: from [Zhang 2005]

While growth rate has been shown to correlate to protein production, the role of oxygen is less consistent. Early works involving protein production in *Pichia* suggest that a dissolved concentration level of 30% is required for protein production, which has resulted in many protocols for recombinant protein production of *Pichia* utilizing the AOX promoter listed 20%-40% DO as the target the methods [Higgins 2007, Invitrogen 2002]. Oxygen is particularly important for *Pichia* grown on methanol, as alcohol oxidase has a high demand for oxygen for the oxidation of methanol [Hartner 2006]. However, some works have demonstrated that decreased oxygen levels and even hypoxic conditions (where oxygen is supplied in limiting quantities) can enhance protein productivity [Baumann 2010]. Furthermore, while some works are able to maintain a constant dissolved oxygen (DO) concentration throughout the fermentation, other works can have increased oxygen demand that surpass the maximum oxygen transfer capabilities of the system and the DO concentration can deviate from this set point [Gurramkonda 2009].

CHAPTER 3: LITERATURE REVIEW

A review of previous works was performed in order to explore what has previously been investigated, with specific attention given to works that utilized *Pichia pastoris* to generate recombinant and/or heterologous proteins using a bioreactor. While continuous cultivations can be used to study production kinetics, the nature of continuous cultivations requires that there be a limiting substrate in order that the dilution rate be matched to growth rate.

In order to find the most appropriate works, the following were used as criteria for selection:

1. **Must be performed in *Pichia pastoris* or a strain formerly known as *Pichia pastoris*:**

While there are many yeast strains available for the production of heterologous proteins, as well as many methylotrophic yeast strains, this analysis will primarily focus on *Pichia pastoris* system, with reference to other yeast systems as appropriate. *Pichia pastoris* was selected due to having significant experimental history as well as a reputation for being a strong producer of proteins. However, with the advancement of molecular genetic tools, a phylogenetic analysis of *Pichia pastoris* resulted in its proposed reassignment to the genus *Komagataella* [Yamada 1995]. Further analysis led to the strain commonly described as *Pichia pastoris* being identified as multiple strains of *Komagataella*, including *K. pastoris*, *K. pseudopastoris*, and *K. phaffi* [Kurtzman 2009]. For this study, any strain that has been given the designation of *Pichia pastoris* or any reassignment thereof, can be used for analysis.

2. **Must be producing a protein with the protein itself being the main product**

While a majority of this work will investigate simple biomass production of *P. pastoris*, the purpose of this work is for the application towards protein production. Recombinant and/or heterologous protein production can be engineered in order to enable cellular production of various chemicals and molecules, such as organic acids and alcohols. Enabling biosynthetic pathways often require cells to synthesize proteins, however, works in which the final target is not protein will not be considered with the same intensity as works where proteins are the final product target.

Protein production can be either intracellular or extracellular, as long as it is the final protein that is the main target product. In some cases, proteins that are produced are enzymes, and the interest is not only in the total amount of protein produced, but also protein that is synthesized (folded) correctly in order to ensure that the protein is in the active form. In order to measure enzyme activity, often these works will quantify production based on the active form of the enzyme, not total protein, and typically use some kinetic unit for quantitative comparison. While these works will be considered, this study will focus more on works that have correlation values that allow for correlating between units per volume to grams per volume (when units are reported).

3. The concentration of methanol in culture/bioreactor needs to be controlled

Growth models are used to relate growth rate to substrate concentration, and therefore accurate measurement of substrate is absolutely necessary. When methanol is used as a substrate, continuous measurement of methanol can be performed via multiple methods; typical methods include automated sampling of fermentation broth and enzymatic measurement of methanol via an offline equipment or, due to the high vapor pressure of methanol, sampling of off-gas via mass spectrometry or IR detection and correlating the amount of methanol in the off-gas to the concentration of methanol in the culture. Any method can be used to monitor methanol; however, the method of methanol data acquisition should be well described in the materials, particularly in terms of temperature and gas flow rate (for off-gas analysis/measurement).

While it is possible for the concentration of methanol to change, depending on feeding methodology, it can be difficult to acquire methanol concertation in sufficient timescale resolution to enable calculations of model parameters under non-steady state conditions. Therefore, for simplicity of calculation, papers in which the concentration of methanol is held constant will be given more attention. This concentration can be zero (i.e. methanol is the limiting factor), but it should remain constant over the entirety of the production phase.

4. **For oxygen data to be included in the analysis, there should be sufficient information regarding oxygen kinetics**

This study seeks to investigate the role/impact of oxygen on *Pichia* growth; therefore, having information on oxygen during growth is necessary. This includes both measurement of oxygen, both in terms of dissolved oxygen as well as oxygen transfer kinetics, and the setup of oxygen control (i.e. aeration rate, gas mixing, method of mixing, agitation range, etc.).

Just as with methanol concentration, oxygen concentration should be measured. While Dissolved Oxygen measurement is typically performed via either polarographic electrode or optical (fluorescing chromophore) probe. While concentration is typically measured in mass per unit volume (such as mg/L or ppm), many factors can affect the oxygen holding capacity of culture media, from osmotic effects to temperature, so it can be difficult to directly measure the concentration of dissolved oxygen. As such, DO is typically measured in terms of percentage dissolved oxygen from a calibration point. While this calibration point is typically the saturation amount of oxygen when ambient air is sparged through the vessel, variations in process conditions such as vessel pressure or gas mixing can shift this calibration point; as such, the description of this calibration point needs to be defined.

While not absolutely necessary for model development, oxygen transfer rate data can also be a valuable metric to capture and include in analysis. Oxygen transfer rate and oxygen uptake rate are important criterion that can be used not only to look at scale up feasibility of limitations, but also to compare between works as OUR is a way to “normalize” the impact of difficult to model variations systems can have on oxygen kinetics, such as reactor geometry, impeller diameter, sparger shape, etc. Should OTR/OUR data be presented, the method for calculation should also be included, particularly if oxygen supplementation is being used. Variations in OTR calculations, like temperature compensation or normalizing mass flow against nitrogen, are important to incorporate in the methods so that consistent calculations can be performed. For example, since OTR can be reported in units of mmol/hr, mmol/(L*hr), or L/hr, specific description of the calculation methods are required to accurately compare works using different units.

5. **Timecourse data should be available for the process parameters, such as Biomass, protein, and substrate concentrations, culture volumes, oxygen transfer rates, temperature, pH, etc.**

In order to calculate production and consumption rates or yields, specific data is necessary. This information can include, but is not limited to, biomass and protein concentration (in g/L), total volume of culture, total volume of feed added, culture or supernatant methanol concentration, dissolved oxygen concentration (either in % ambient or ppm), and oxygen transfer/uptake rates (in units g/(L*hr) or mol/(L*hr)). Furthermore, sufficient description should be presented on how the instruments were calibrated to obtain the online information, or how the calculations and considerations used to obtain calculated values.

It is of note that capturing this data, while being valuable for process development, may not be within the scope of the original work or is difficult to capture and as such many of these values are not included in the data or figures of the manuscripts (even in the supplementary material available online). While this does not detract from the scientific merit of these works, including this data would enable more profound and robust investigations into the impact of process parameters on rProtein production.

6. **Works can include multiple stages; i.e. a stage where a non-inducing carbon source is added to increase biomass prior to induction**

The most “simple” form of cell culture for production in terms of operation is often considered “batch culture” where the culture is in a “production phase” when the process is initiated and nothing is added to the culture until termination; this is due to the minimal intervention by the user. Performing fed-batch culture increases the complexity as this requires additional equipment for adding materials as well as consideration of material addition schema/methods. Fed-batch allows for continuous addition of carbon source, increasing the amount of carbon that can be converted to product. This is particularly important for carbon sources or nutrients that can be harmful for microbial fitness at increased concentrations, such as ethanol and methanol.

With fed batch culture, it is possible to change the addition schedule/pattern and shift carbon source, such as initially feeding with a non-inducing carbon source and then shifting to an inducing

carbon source. For *Pichia* using the AOX promoter system, the non-induced "biomass production" phase is typically accomplished by feeding either glucose or glycerol and then the culture is shifted to a "production" phase where methanol is added. This "dual-phase" production scheme can be particularly useful when protein production or the protein being produced negatively impacts microbial fitness, as this allows for more biomass to accumulate and be available before switching to production.

CHAPTER 4: DEVELOPMENT OF BI-VARIATE MODEL FOR GROWTH RATE IN *PICHIA PASTORIS*

Multi-substrate models have been developed and implemented for other organisms and protein production; including hybridoma cells utilizing glucose and glutamine as substrates producing immunoglobulins (IgG) [Bree 1988] and *Pseudomonas putida* grown on phenol and oxygen [Şeker 1997]. While these models were developed for carbon and nitrogen components, *Pichia* growth and production models could be updated to incorporate both methanol and oxygen, which would allow for application to both methanol and oxygen. Dual substrate models for *Pichia* on glycerol and oxygen have been applied previously in order to improve controller performance [Oliveira, 2005] as well as for *Pichia* grown on methanol/glycerol mixtures [Canales 2018]. Applying this same methodology to methanol and oxygen as substrates, the model becomes Equation 1.

Equation 1 (E1):

$$\mu = \mu_{max} \cdot \left(\frac{[Me]}{K_{Me} + [Me]}\right) \cdot \left(\frac{[O_2]}{K_{Ox} + [O_2]}\right)$$

While kinetic data on oxygen requirements in *Pichia* is limited, work by [Ponte 2016] demonstrates the dependence of on growth rate and protein production on oxygen concentration, as well as the potential impact of increase levels of oxygen on recombinant protein production. However, other modeling factors have been proposed based on various biological phenomena and considerations, including maintenance coefficients (a term used to describe substrate that is used for neither biomass production nor product formation) as well as substrate inhibition. The apparent shift in the growth kinetics can be accounted for by incorporation of maintenance coefficient term; at increased biomass concentrations, maintenance coefficient can equal 10%-15% of the substrate yield. Accounting for maintenance coefficient (as a threshold substrate, [Frame 1991]) for oxygen results in the Equation 2:

Equation 2 (E2):

$$\mu = \mu_{max} \cdot \left(\frac{[Me]}{K_{Me} + [Me]}\right) \cdot \left(\frac{[O_2] - [O_{2,t}]}{K_{Ox} + [O_2] - [O_{2,t}]}\right)$$

Furthermore, the model can also include terms for substrate inhibition (when the substrate is at increased levels and causes a decrease in cellular growth) such as for the observed inhibitory effects of increased concentrations of methanol [Barrigon 2015] as well as oxygen [Ponte 2016]; however, multiple forms for substrate inhibition have been proposed. A nonlinear equation was proposed by Levenspiel accounted for the influence of product ethanol on growth rate [Levenspiel 1980]. Incorporating this form of for both substrates as well as including the substrate threshold term for maintenance for oxygen, the model then becomes Equation 3:

Equation 3 (E3):

$$\mu = \mu_{max} \cdot \left(\frac{[Me]}{K_{Me} + [Me]}\right) \cdot \left(1 - \frac{[Me]}{K_{I2,Me}}\right)^{n_{Me}} \cdot \left(\frac{[O_2] - [O_{2,t}]}{K_{Ox} + [O_2] - [O_{2,t}]}\right) \cdot \left(1 - \frac{[O_2] - [O_{2,t}]}{K_{I2,O_2}}\right)^{n_{O_2}}$$

However, this equation is based on ethanol being formed as a product. Furthermore, this model implies that there is a critical substrate value at which any further increase in substrate results in full retardation of growth; while this phenomena may be appropriate for alcohol substrates, such as methanol and ethanol, typical dissolved oxygen concentrations seen during a *Pichia* fermentations are not high enough to fully stop growth. A non-monotonically increasing function for growth rate incorporating inhibition based on substrate inhibition was proposed and successfully applied specifically to *Pichia* and methanol [Barrigon 2015]. This form the inhibition term has also been attributed to describing the inhibitory effect of high O2 concentrations on cell growth during anaerobic digestion [Villadsen 2011]. Utilizing this non-monotonic form of the inhibition term factor instead results in Equation 4:

Equation 4 (E4):

$$\mu = \mu_{max} \cdot \left(\frac{[Me]}{K_{Me} + [Me] + \frac{[Me]^2}{K_{I,Me}}} \right) \cdot \left(\frac{[O_2] - [O_{2,t}]}{K_{Ox} + [O_2] - [O_{2,t}] + \frac{\left[[O_2] - [O_{2,t}]\right]^2}{K_{I,Ox}}} \right)$$

Furthermore, both forms of inhibition terms can be applied combinatorially, similarly to a model applied to the production of ethanol by *Scheffersomyces stipitis* from xylose [Farias 2014]. Integrating both terms results in the growth equation for each substrate leads to Equation 5 and Equation 6. Table 2 contains a summary of these equations.

Equation 5 (E5):

$$\mu = \mu_{max} \cdot \left(\frac{[Me]}{K_{Me} + [Me] + \frac{[Me]^2}{K_{I,Me}}} \right) \cdot \left(\frac{[O_2] - [O_{2,t}]}{K_{Ox} + [O_2] - [O_{2,t}]} \right) \cdot \left(1 - \frac{[O_2] - [O_{2,t}]}{K_{I2,O_2}} \right)^{n_{O_2}}$$

Equation 6 (E6):

$$\mu = \mu_{max} \cdot \left(\frac{[Me]}{K_{Me} + [Me]} \right) \cdot \left(1 - \frac{[Me]}{K_{I2,Me}} \right)^{n_{Me}} \cdot \left(\frac{[O_2] - [O_{2,t}]}{K_{Ox} + [O_2] - [O_{2,t}] + \frac{\left[[O_2] - [O_{2,t}]\right]^2}{K_{I,Ox}}} \right)$$

Experimental data would be required to validate which form of the bi-variate equation is more closely correlated to *Pichia* growth, however, plotting the bivariate inhibitory model for growth rate utilizing the Levenspiel Inhibition form for methanol and the Substrate Inhibition form for oxygen (E6) as well as using arbitrary values for the constant terms produces a surface (Figure 5a, Figure 5b) where multiple combinations of parameters result in similar growth rate (Equation 2). The values used for constants in creating this curve were selected based on their correlation to previous works [Barrigon 2015, Ponte 2016] and will be discussed more extensively in Chapter 6.

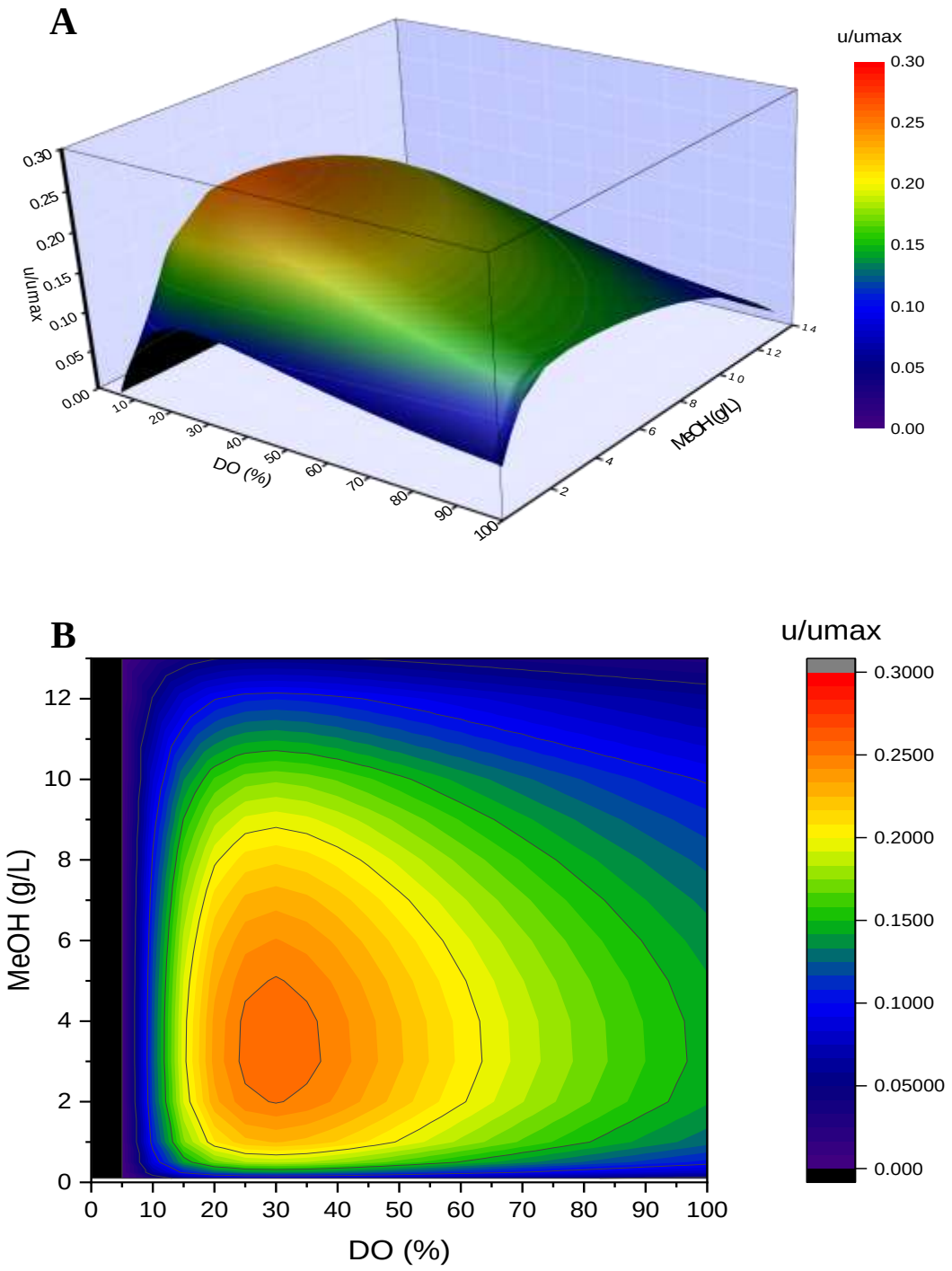

Figure 5: Surface plot of bivariate *Pichia* growth model against methanol and dissolved oxygen; A) 3-dimension surface plot and B) 2-dimensional projection of surface; black lines indicate culture conditions that theoretically would result in equivalent growth rates. Values for this image were calculated using Excel (Microsoft, 2013) and the images generated using Origin Imaging Software (OriginLab Corp, 2018)

Again, previous works have often correlated/attributed growth rate as an influential factor in protein production in *Pichia* with many discussions and analyses of protein production by *Pichia* utilizing the AOX promoter report optimal protein production based on growth rate alone [Barrigon 2015, Looser 2015, Prielhofer 2018, Peebo 2018] or utilize growth rate as the control variable for metabolic studies [Rebnegger 2014]. However, under multidimensional design space, multiple combinations of parameters with dramatically different operational requirements could result in equivalent growth rates that do not translate into equivalent protein production.

This model encourages the multiple questions: Is protein production solely a function of growth rate, or do similar growth rates under various component/nutrient limitation result in varied protein production? Furthermore, does the cell respond to various nutrient limitations in a way that could inform which type of nutrient limitation (and therein, which type of feed profile) would be most effective for the protein being produced? Lastly, once more data is available, which form of the inhibitor term is more appropriate, can the selection of an inhibitory term further inform the mechanism of inhibition by methanol and oxygen?

Table 2: Summary of variations of bi-variate growth equations for *Pichia pastoris* on methanol and oxygen

Equation	Methanol Term Method	Oxygen Term Method	Equation
E1	Monod	Monod	$\mu = \mu_{max} \cdot \left(\frac{[Me]}{K_{Me} + [Me]}\right) \cdot \left(\frac{[O_2]}{K_{Ox} + [O_2]}\right)$
E2	Monod	Monod Threshold	$\mu = \mu_{max} \cdot \left(\frac{[Me]}{K_{Me} + [Me]}\right) \cdot \left(\frac{[O_2] - [O_{2,t}]}{K_{Ox} + [O_2] - [O_{2,t}]}\right)$
E3	Monod Levenspiel Inhibition	Monod Threshold Levenspiel Inhibition	$\mu = \mu_{max} \cdot \left(\frac{[Me]}{K_{Me} + [Me]}\right) \cdot \left(1 - \frac{[Me]}{K_{I2,Me}}\right)^{n_{Me}} \cdot \left(\frac{[O_2] - [O_{2,t}]}{K_{Ox} + [O_2] - [O_{2,t}]}\right) \cdot \left(1 - \frac{[O_2] - [O_{2,t}]}{K_{I2,O_2}}\right)^{n_{O_2}}$
E4	Monod Substrate Inhibition	Monod Threshold Substrate Inhibition	$\mu = \mu_{max} \cdot \left(\frac{[Me]}{K_{Me} + [Me] + \frac{[Me]^2}{K_{I,Me}}}\right) \cdot \left(\frac{[O_2] - [O_{2,t}]}{K_{Ox} + [O_2] - [O_{2,t}] + \frac{[[O_2] - [O_{2,t}]]^2}{K_{I,Ox}}}\right)$

Table 2 continued

E5	Monod Substrate Inhibition	Monod Threshold Levenspiel Inhibition	$\mu = \mu_{max} \cdot \left(\frac{[Me]}{K_{Me} + [Me] + \frac{[Me]^2}{K_{I,Me}}} \right) \cdot \left(\frac{[O_2] - [O_{2,t}]}{K_{Ox} + [O_2] - [O_{2,t}]} \right) \cdot \left(1 - \frac{[O_2] - [O_{2,t}]}{K_{I2,O_2}} \right)^{n_{O_2}}$
E6	Monod Levenspiel Inhibition	Monod Threshold Substrate Inhibition	$\mu = \mu_{max} \cdot \left(\frac{[Me]}{K_{Me} + [Me]} \right) \cdot \left(1 - \frac{[Me]}{K_{I2,Me}} \right)^{n_{Me}} \cdot \left(\frac{[O_2] - [O_{2,t}]}{K_{Ox} + [O_2] - [O_{2,t}] + \frac{[[O_2] - [O_{2,t}]]^2}{K_{I,Ox}}} \right)$

CHAPTER 5: CURRENT FEEDING METHODS; IMPACT OF PROCESS CONDITIONS ON GROWTH AND PROTEIN PRODUCTION

Since growth rate has been shown to affect recombinant protein production, fed-batch have the advantage of allowing for control of carbon addition rates which can be used to directly control growth rates. However, there are numerous types of feeding methodologies, each with its own degree of complexity to implement and necessary equipment to perform. Many fermentation feeding methods were developed on non-product forming strains, where biomass is the target product. As such, product formation can have a significant impact on culture kinetics, such as carbon allocation or product toxicity, and lead to growth cessation independent of feeding methodology. While product formation can and often does affect culture kinetics, it is important to consider the developmental origin of a feed strategy in order to consider if other strategies may be appropriate.

As stated before, the feed of the fed-batch processes consist of a concentrated source of carbon that is fed into the reactor. Feeding methodologies typical fall into two categories: feed-forward methods (also called open loop control) and feed-back methods (also called closed loop control). Selection of feeding methodology for a fed-batch process can be based on any number of parameters; the increased price and limited availability of costly sensors can limit the usability of feed-back methods, while lack of growth and product formation kinetic data can limit the prediction capabilities and usability of feed-forward methods. Feed strategies can also be selected based on preceding works or based on demonstrated success in previous growth and production trials.

The following sections provide an overview of select feeding methods common to fermentation operations. This list is certainly not exhaustive, but is meant to serve as a starting point for further discussion on feeding methods as well as concise ‘inspiration’ for other feeding methods that can be explored. While other works have specifically investigated the growth kinetics of industrially standard protocols [Looser 2015] this work will look at general feed strategies that have been applied to a wide range of organisms which may be of interest for *Pichia* fermentations.

6.1: Feed-Forward methods:

Feed-forward methods control action from a controller independently of process outputs; typically in the form of a pre-programmed feed schedule. Often, these feeding methods can be implemented without the need for complex and expensive sensors and probes. Furthermore, since these feeding methods are open-loop (preprogrammed), the feeding schedule, and therefore the overall feeding rate and feed amount, are not influenced by culture conditions and can be consistent, removing a possible source of variability across experimental treatments in terms of feed input.

The most straightforward fed batch operation is the ‘constant flow’ feeding method [Parashar, 2016, Kim 2018, Liu 2018]. In this feeding method, constant feed rate is maintained. Once the batch carbon is completely utilized, this feeding method limits growth rate. As constant feeding continues, the biomass increases, resulting in more substrate requirement, limiting the specific growth rate. As such, specific growth rate decreases over the course of a constant fed batch growth.

The next feeding method is the ‘linear increasing’ feeding. In this feeding method, an initial feed rate is selected, but the feed is allowed to constantly increase over time [Hou 2017]. Since feeding rate is able to increase over time, growth rate is also able to initially increase (as compared to constant feed). However, due to the linear nature of the feed combined with the exponential nature of cell growth, the growth rate will plateau and then begin to decrease over time.

Similar to the linear increasing feed method is the stepped increasing feed method [Chen 2017, Li 2018]. With this feeding method, the feed rate is not increased constantly over time, but increased in larger steps after a certain periods of time. When the feed rate increases, there is a sharp increase in growth rate followed by a steady decrease in growth rate during each period of constant feeding. However, the overall growth rate trend is similar to that of the linear increasing feed, but with sharp jumps in growth rate.

The ‘timed pulse’ feeding method is a form of intermittent feeding, where large bolus amounts of feed are added to the reactor based on a pre-determined schedule in evenly space intervals [Sun 2016]. This feeding method is different in that feed is not added constantly over the entire duration; the feed is added such that excess substrate is added, which is consumed in the period between

pulses. During this type of feeding, cells grow at maximum growth rate in the presence of excess substrate until all the substrate is consumed. Once the substrate is consumed, cells will move into endogenous metabolism in order to survive until the next bolus of substrate (this can be accompanied by a decrease in biomass due to maintenance nutrient requirements). As biomass increases, the amount of time the culture experiences starvation condition increases, until the point where all the substrate is consumed during the feeding of the bolus.

Another form of feeding method is the “exponential” feeding method. This feeding method takes advantage of the exponential characteristic of microbial growth. Substrate feeding is increased in an exponential rate, which allows for cells to grow at a constant specific growth rate. While this feeding method can give a fairly constant growth rate, precise considerations of substrate requirements, such as cellular maintenance terms [Vos 2016] or volume changed associated with culture sampling, must be included in order to accurately control specific growth rate. As stated before, growth rate can have a profound impact on culture kinetics and as such, exponential type feeding can be a powerful feeding method [Singh 2012]. It can be used to generate increased biomass without accumulation of metabolic byproducts, or to maintain an optimum growth rate for protein production. This type of feeding can be programmed directly (using an equation where the starting feed is multiplied by an exponent over time) or by simply varying the feed rate directly with the volume (i.e. Feed rate = X “mL/L/hr”) [Yu 2017].

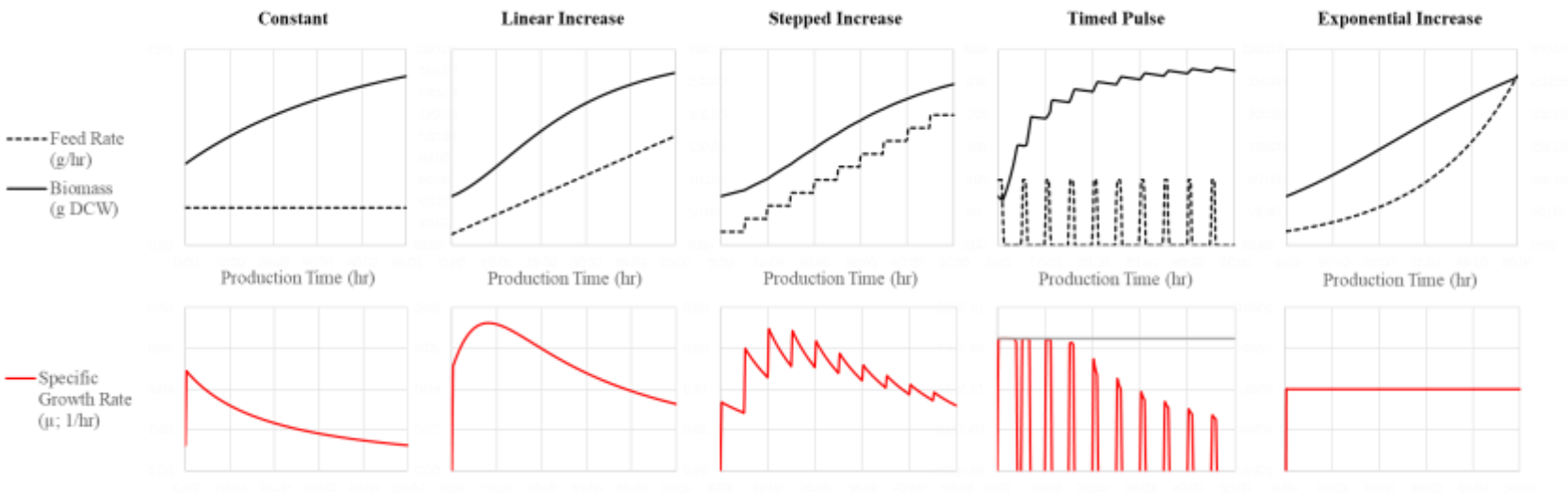

Figure 6: Feed rate with hypothetical Biomass, and growth rate of various feed-forward feeding methods. While the units of these graphs are arbitrary, in the event sufficient data is known correlating growth rate and protein productivity, these trends can be useful for selecting a feeding strategy for investigation. These trends were developed in the Maple computing environment [Maplesoft, 2018]. The equations and code used to create these can be found in Appendix A

Since substrate feed rate is calculated/defined prior to growth occurring if sufficient information is known about the growth characteristics of a strain, such as yield and maintenance constants, predictions can be made about the culture kinetics. While there are limitless combinations of process parameters that can result in unique growth rates and trends, exploring general trends of biomass formation prior to experimentation can be beneficial. The trends in Figure 6 were developed based on the feed forward profiles, growth kinetic equations, and various (arbitrary) growth parameters. The MAPLE math engine software was used to combine Ordinary Differential Equations for feed and growth information and approximated using 4th-5th order Runge-Kutta-Fehlberg method. While these trends are artificial and do not take into account the effects of protein production on culture growth, they demonstrate the general effect of various feeding methods on growth rate.

For example, if a specific growth rate has been shown to produce that maximum protein production/productivity, the exponential feed strategy (Figure 6: Exponential) could be advantageous as this feeding methods maintains a constant growth rate over the course of the fermentation (at least until a substrate becomes limiting). Linearly increasing feeding is also a commonly used feeding method (Figure 6: Linearly Increasing), but the operator should be aware that there will be an initial increase in growth rate as the linearly increasing feed over takes the exponentially increasing cells; in the event higher growth rates are disadvantageous to culture performance, this phenomena should be considered and mitigated if need be.

Feed-forward feeding methods, by design do not respond to culture conditions and can be performed invariable in regards to feeding kinetics. However, this does not mean that culture response will be consistent. Any change in carbon utilization or metabolic flux can lead to fluctuations biomass in carbon availability. Variations in cell culture genetics can lead to variation in cell performance (product formation, metabolic overflow, byproduct formation, etc.) when treated with the same feed rate. Thus, using pre-defined feed-forward methods between different strains, constructs, or products can produce biased results. This consideration should be taken into account when using feed-forward methods for construct screening. Furthermore, the trends listed in table 1 are based on the feeding substrate being the limiting substrate; as growth proceeds, other nutrients may become the limiting substrate that is not the fed substrate. Limitations such as

Oxygen Transfer, batch media nutrient exhaustion, and even simple volume limits can cause deviations from these predicted trends. Once these limits are reached, a different feed strategy may be required or the fermentation be terminated. These limits can become even more pronounced during scale up efforts and therefore should be considered when selecting a feed method at smaller volume/scale.

6.2: Feed-Back methods:

Feed-back methods control feeding action based on process outputs, typically from signals outputted from sensors monitoring process parameters to some type computation unit. Since these methods respond to process outputs, they all require some type of sensor as well as some form of signal conditioning unit, such as a PID controller or logic controller, in order to take the feed signal and make modifications to the feed. For all Feed-Back methods, control theory is directly applicable where control systems engineering can result in a more predictable and/or robust (or desired) response. Furthermore, a detailed understanding of the sensor capabilities and limitations (such as sample rate, chemistry interactions, calibration drift, etc.) is necessary for accurately interpreting the response signal.

Likely the most straightforward feed-back method is the direct sensing of substrate and modifying the feed based on this signal. Many different types of sensors have been developed and are available, from direct sensing in culture media [Pimentel 2018)] or offgas [Güneş 2016], withdrawing sample for analysis [Chongchittapiban 2016], or sensing via spectroscopy [Goldfeld 2014], each with its own chemistry and sampling method. This form of fed-batch control is the most straightforward application of control theory, with major factors including sample time and sensor delay as well as the algorithm applied. Furthermore, by the nature of the probe sensing a substrate, this control requires that substrate must be excess in the culture media, or else the probe will simply read a null value. Depending on the logic of the controller, this “bottoming out” of the parameters can cause complications in terms of smooth operations, leading to dramatic swings or “kick back” in substrate concentration.

Next to carbon, oxygen is the second most common molecule in biomass and is therefore an important molecule for fermentation, especially for aerobic fermentation. Since oxygen is a vital

component of biological processes, dissolved oxygen levels during fermentation can be a key process parameter. Ambient air and/or mixtures of pure oxygen are sparged into the reactor. Only oxygen dissolved in the aqueous media is considered bio-available however and since oxygen has a very low solubility (when compared to other chemicals), its maximum dissolved concentration is typically on the level of mg's/L. In aerobic fermentations, carbon consumption is generally accompanied by dissolve oxygen uptake. When oxygen uptake is greater than oxygen transfer, dissolved oxygen levels decrease. Oxygen uptake is typically a function of cellular metabolic functions while oxygen transfer is a function of control parameters, such as air flow, impeller and tank geometry, agitation speed, etc. Once carbon (or any substrate) is fully utilized, oxygen requirement and subsequent uptake decrease, resulting in an increase in dissolved oxygen level. DO-stat or DO-pulse feeding methods are triggered by these sudden increases in oxygen (typically by a level threshold) as an indication that substrate has been utilized and the more substrate is added to the reactor. Typically the timescale of DO-stat response, from substrate exhaustion to substrate replenishment, is much faster than pH-stat methods. As stated before, longer periods of starvation are associated with shifts in metabolic profile; this may be a consideration when selecting a feeding method. Furthermore, consideration must be given to oxygen transfer capabilities of aerobic functions. OTR at 20% dissolved oxygen level at 400rpm is significantly different than 20% dissolved oxygen and 1500rpm. DO based feeding has been successfully used in producing proteins from a wide range of organisms, including *Pichia pastoris* [Picotto 2017, Ferreira 2012].

Beyond oxygen, analyzing various system gasses can be also be analyzed to gain further insight into the status of the fermentation, as well as become a method for controlling a fermentation. As stated with DO-stat feeding, Oxygen Transfer rate can be calculated from oxygen concentrations from inlet and outlet. Equivalent CO2 measurements can be to calculate Carbon Dioxide Evolution Rate (CER), a parameter which can be used to track carbon allocation and obtain data about oxidative balances within the cell. For processes that use or produce volatile species, such as alcohols, the concentration of these volatile species in the fermentation media can often be determined based on the concentration in the exhaust gas. All of these measurements /calculations can be used to modify feed. Overall, feedback methods are powerful in that they are responsive to the system; this feature can enable each individual cell lines constructs to respond individually to

as process, but still maintain consistent process parameters. However, these feeding methods can also be unstable; should the probe malfunction or an unforeseen event occur, the culture will respond to these errors, possibly to a detriment to the experiment which can require a repeat and further used of time and resources.

6.3: Combined and Advanced Methods:

One powerful aspect of fed-batch control is the ability to separate the process into different phases and have different feeding methods for each phase. For example; for production proteins that have a detrimental effect on cellular functions, such a proteases and lipases which can destroy key cellular parts, initiating protein synthesis from the start of a process could lead to overall decreased productivity as cells fitness is negatively impacted by the protein produced. As such, it may be beneficial to separate the process into two phases, a first phase where biomass is accumulated and a second phase where protein is produced. Since the two phases each have a different purpose, two different feeding methods can be used to target the different objectives of the two phases.

6.4: Manually adjusted Feeding:

It is worth mentioning that many works utilize a "manual feeding" method, where a feed is modified based on the user's discretion [Zhang 2017]. While circumstances may necessitate the use of this feeding method, it is the opinion of the author that this method be avoided. Factors such as variable time between samplings, variations in human judgment, and varying levels of user experience can lead to lack of reproducible results. Unless the methodology for sample analysis and result response is clearly defined and strictly adhered to, manual feeding can cause discrepancies between treatments. Should manual feeding be required, sufficient explanation or discussion should be given in order to allow for reproducible results (or another feeding method should be utilized).

CHAPTER 6: ALIGNMENT OF BI-VARIATE MODEL WITH PREVIOUS WORKS

Significant work has been performed in order to model the growth of, and subsequent production of proteins, by *Pichia* with unstructured models, often based on or combinations of equations developed by Monod, Haldale, Pirt, and Ludeking-Piret. Growth rate in particular has been shown to regulate many aspects of yeast physiology, including genome wide regulatory changes and stress response [Rebnegger 2014]. Furthermore, protein productivity has been shown to be related with growth rate, particularly for the AOX promoter, in which optimal protein production occurs at a growth rate below μmax [Zhang 2005]. An unstructured macrokinetic model was developed for *Pichia* producing a Rhizopus oryase lipase (ROL) [Barrigon 2013, Barrigon 2015], which demonstrated the growth rate dependence of recombinant protein production under AOX. This model included values for accounting for saturation kinetics as well as values accounting for cell, substrate, and product inhibition (non-monotonically increasing function).

Due to the toxic and growth inhibitory nature of methanol as a substrate at increased concentrations, protein production is often performed using fed-batch operations. The feeding methodology of fed-batch productions of recombinant proteins using *Pichia pastoris* typically fall into one of three schema; A) feeding rate is modified in order to maintain a steady target concentration of methanol in the culture [Gurramkonda 2009] B) the feed rate is controlled to maintain a constant growth rate C) the feed rate is modified based on other parameters, such as dissolved oxygen [Ferreira 2012, Picotto 2017] or temperature [Jahic 2003]. The first two are direct applications of the developed growth models; a concentration of methanol is selected to target a growth rate or methanol is fed at an exponentially increasing rate in order to maintain a target growth rate with the growth rate such that it results in maximum protein production and productivity. A mathematical model of *Pichia* was successfully applied to optimize substrate-feeding method [Ponte 2018] allowing for a 2.2 fold increase in final protein production compared to pre-programmed exponential feeding methods demonstrating the value of these models.

While significant efforts have been made to model the inhibition kinetics of alcohol on yeasts [specifically ethanol, Luong 1985] as well as to model the effects of methanol on *Pichia* growth

and protein production [Barrigon 2015, Looser 2015, Zhang 2000], few works could be found that look specifically targeted modeling the effects of oxygen on *Pichia*. Previous models have already demonstrated interesting potential using previous generated data [Barrigon 2015, Ponte 2016] but were only applied for methanol. This work proposes various models and modelling terms in order to encourage the exploration of oxygen effect on growth and protein production in *Pichia.* Data for growth rate based on oxygen and methanol concentration for *Pichia* grown is available [Barrigon 2015, Ponte 2016]. These works were able to be confidently combined as both the same strain (*P. pastoris X-33*), expression vector (pPICZαAROL), and target protein (lipase from *R. oryzae*) were the same between both works. Using the data from these works simultaneously, Equation 6 of the bivariate model was applied to incorporate both oxygen and methanol data:

Table 3: Macrokinetic Models parameters for *Pichia* growth on methanol for various growth models

		Bi-Variate model (Eq. 6)	Non-monotonically increasing function	Monotonically increasing function
Model Parameter	Units	This Work	Barrigon 2015	Barrigon 2015
μ_{max}	hr^{-1}	0.17	0.069	0.042
K_{Me}	g/L	0.2	0.4	0.1
$K_{I,Me}$	g/L	----	8.85	----
$K_{I2,Me}$	g/L	15	----	----
n_{Me}	----	0.4	----	----
$O_{2,t}$	%	5	----	----
K_{Ox}	%	30	----	----
$K_{I,Ox}$	%	17.5	----	----

While sufficient data is not available to fully validate which form of the equation would be most accurate, the values selected here show strong alignment with the empirical data obtained previously. Based on these considerations, Equation 6 was ultimately selected for investigation as having the most potential to accurately model both methanol and oxygen. As such, Equation 6 was used as the basis for this works investigation platform and therefor these terms were optimized in order to demonstrate its potential; this single, bivariate, dual-substrate model shows strong

consistency with both methanol and oxygen data from both works simultaneously (Figure 7a, Figure 7b).

Further investigations which include both oxygen and methanol in the growth model could lead to insight into *Pichia* growth and production as the various forms of the model and their factors are validated. First off, the "Levenspiel" form of inhibition (Equation 3) is such that ultimately there is a critical substrate value after which the inhibition is so strong that growth in completely inhibited. While this phenomena can be expected for high alcohol concentrations, growth in *Pichia* has been consistently observed well up to complete oxygen saturation. This means that the "Levenspiel" form of inhibition is not appropriate for the oxygen inhibition considerations for *Pichia* growth models. Conversely, the non-monotonic, substrate inhibition form, while applied in Barrigon, may in fact not be an accurate model term. In work by Barrigon, this non-monotonic form for methanol inhibition was shown to be more accurate in predicting growth and protein production in *Pichia* when comparted to monotonically increasing function (i.e. the Monod equation). However, when this non-monotonically increasing function is expanded to higher methanol concentrations than what were used experimentally, the term predicts that there will never be a high enough concentration of methanol that completely inhibits growth, which in highly unlikely.

Equation 6 includes a term which accounts for the maximum concentration of methanol at which growth ceases ($K_{I2,Me}$). While the value used in this work (15 g/L) is lower than what has been shown for growth in other studies ([Zhang, 2000] demonstrated growth in methanol greater than 25 g/L), this discrepancy could be an artifact of the different proteins being produced between the different works. Clearly, the limited number of data is not sufficient for definitely validating which version of the bivariate inhibitory model is most appropriate, however this uncertainty can motivate further investigation into *Pichia* physiology and protein production mechanisms: does *Pichia* have mechanisms to withstand higher methanol concentrations? Is this mechanism disrupted by protein production? Etc. Further testing would be required in order to validate the model and in validating this model, a more profound understanding of *Pichia* physiology and metabolism would be gained. Furthermore, there is only enough data to model growth rate and not protein production. However, as stated in Chapter 4, this work is an effective starting point to truly

identify if growth rate alone dictates protein productivity, or there are in fact other biological phenomena that are more influential in protein productivity.

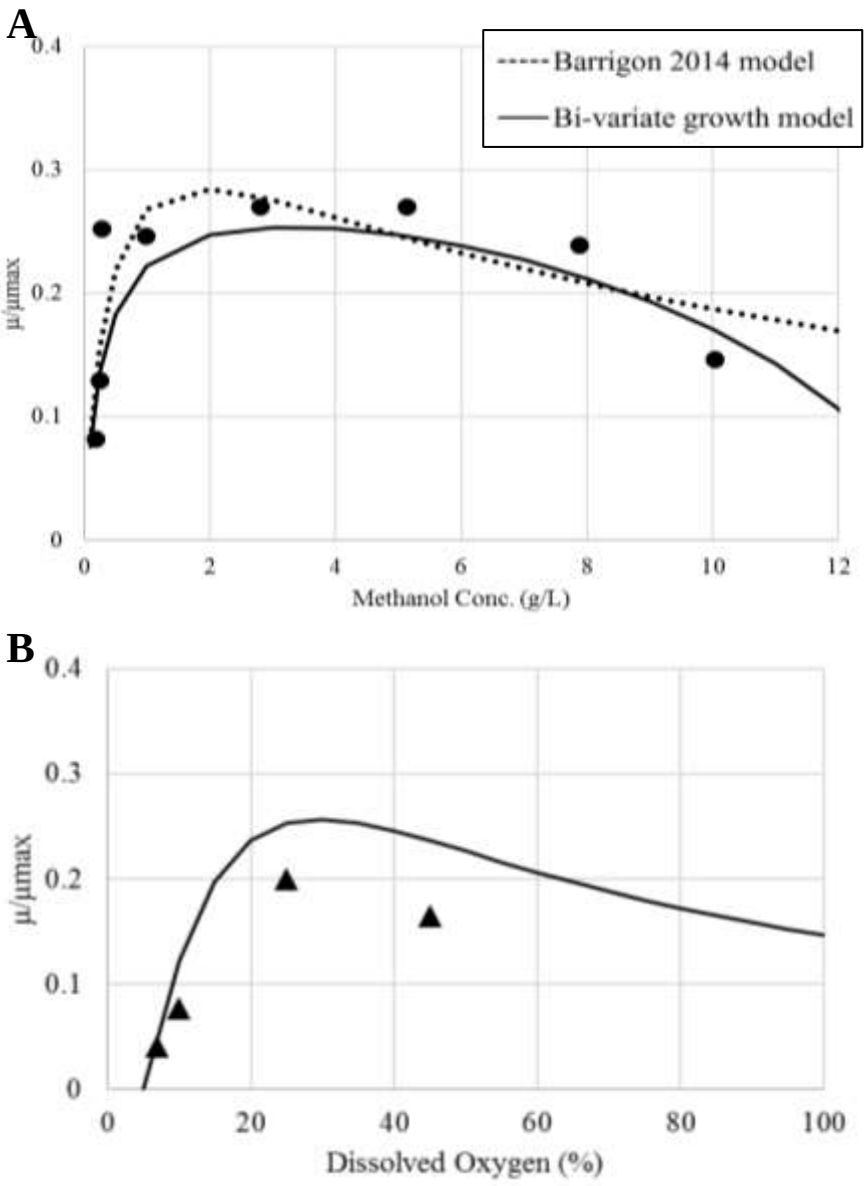

Figure 7: Bi-variate growth model approximations superimposed on other works; Black line: Bi-variate growth Equation 6 [This work] utilizing model parameters from Table 3; Black Circles: Empirical data from [Barrigon 2015]; Dotted line: Non-monotonically increasing function from [Barrigon 2015]; Black Triangles: Empirical Data from [Ponte 2016]

CHAPTER 7: SUMMARY OF POTENTIAL IMPACT ON CURRENT PARADIGM AND NEXT STEPS

While there is insufficient data publically available to definitive identify the most appropriate model equation for oxygen considerations, this work and the models and considerations presented herein encourage the inclusion of oxygen and oxygen kinetics and as such provide an initial framework in which to enable this investigation to occur.

As stated previously, in a two dimensional system, the model development in this work propose that numerous combinations of environmental conditions (from methanol and dissolved oxygen concertation) will result in similar or comparable growth rates, even under dramatically different environments. Based on the current paradigm of recombinant protein production in *Pichia pastoris* using the AOX promoter system (in which protein productivity can be correlated to growth rate), any combination of parameters which result in comparable growth rate should also result in comparable protein productivity metrics. These combinations of parameters though can vary though, and even if they result in similar overall growth rates, it is likely that the metabolic processes and overall "state" of the cells will be different, as the cell will be experiencing different degrees of nutrient limitation and substrate inhibition. It is also possible that the varied metabolic state of the cell, even at similar growth rate, can have a direct impact on protein production and productivity. Furthermore, the extent to how protein production is affected could be different between various proteins. Prior to investigating how the various combinations of methanol and oxygen affect growth and protein production, and therein verifying if protein productivity is solely correlated to growth rate, a framework is necessary in order to guide and robustly analyze and compare works. Overall, this work encourages a more comprehensive viewpoint for *Pichia* fermentations as well as fermentation investigations as a whole and effectively challenges the current paradigm of recombinant protein production in *Pichia*, where rProtein productivity is linked to growth rate.

Not only does this provide the initial framework to investigate combined effects on methanol and oxygen limitation/surplus on *Pichia* metabolism and protein productivity, but the bi-variate model can also provide a structure for allowing further investigations into feeding methodologies that are

used for AOX based *Pichia* fermentations. As stated previously, feed forward methods can and are often used in order to target specific growth rates and growth profiles, which can be advantages for *Pichia* fermentations where growth rate has been shown to correlate with protein productivity. However, extensive pre-investigative work is required in order to identify the necessary growth rates and feeding profile for production since protein yields and the impact of protein production on growth can differ dramatically between proteins. Feed-back methods have the advantage in that the feeding methodology can respond to the various discrepancies between culture performances and maintain similar or comparable process parameters. However, current models are only based on methanol and therefore only methanol can be used as a process control parameters and contextualized using models. Because of this, feed-back methods are generally limited to only controlling methanol (typically holding methanol constant); any other derivations cannot be accounted for in the model. This is especially significant for oxygen since oxygen transfer can be one of the major physical limitations of a system, not only at research level scale, but also at industrially relevant scales. Therefore development of a bi-variate model can enable more robust control and analysis of fermentation experiments, especially those at that occur near the physical limits of the system.

In order to effectively implement the bi-variate model for *Pichia* including both methanol and oxygen, first, growth experiments would need to be performed similar to Barrigon and Ponte, but at increased oxygen and methanol concentrations in order to identify the most appropriate form of the inhibition term (i.e. non-monotonically increasing function vs. “Levenspiel” form of inhibition term). It would also be beneficial to perform these experiments under producing and non-producing circumstances (non-producing = empty vector) in order to identify the impact of protein production on the growth models. Once the most appropriate version of the bi-variate form of the growth model can be identified, trails can be initiated which investigate and confirm the correlation between growth rate and protein productivity in an expanded design space. This bi-variate model could then be used as the framework under which metabolic studies (metabolomics, transcriptomics, etc.) could be applied to and aligned with process development work with the ultimate goal on enabling enhanced protein production capabilities in *Pichia*/AOX systems and/or decreased experimental timelines.

REFERENCES

Ahmad, M., Hirz, M., Pichler, H., & Schwab, H. (2014). Protein expression in *Pichia pastoris*: recent achievements and perspectives for heterologous protein production. Applied microbiology and biotechnology, 98(12), 5301-5317.

Arkinstall, S., Edgerton, M., Payton, M., & Maundrell, K. (1995). Co- expression of the neurokinin NK2 receptor and G- protein components in the fission yeast *Schizosaccharomyces pombe. FEBS letters*, 375(3), 183-187.

Barrigón, J. M., Montesinos, J. L., & Valero, F. (2013). Searching the best operational strategies for *Rhizopus oryzae* lipase production in *Pichia pastoris* Mut+ phenotype: Methanol limited or methanol non-limited fed-batch cultures?. *Biochemical engineering journal*, 75, 47-54.

Barrigon, J. M., Valero, F., & Montesinos, J. L. (2015). A macrokinetic model- based comparative meta- analysis of recombinant protein production by *Pichia pastoris* under AOX1 promoter. *Biotechnology and bioengineering*, 112(6), 1132-1145.

Baumann, K., Maurer, M., Dragosits, M., Cos, O., Ferrer, P., & Mattanovich, D. (2008). Hypoxic fed- batch cultivation of *Pichia pastoris* increases specific and volumetric productivity of recombinant proteins. *Biotechnology and Bioengineerin*g, 100(1), 177-183.

Baumann, K., Carnicer, M., Dragosits, M., Graf, A. B., Stadlmann, J., Jouhten, P., ... & Ferrer, P. (2010). A multi-level study of recombinant *Pichia pastoris* in different oxygen conditions. *BMC systems biology*, 4(1), 141.

Boidin, A., & Effront, J. (1917). U.S. Patent No. 1,227,374. Washington, DC: U.S. Patent and Trademark Office.

Bree, M. A., Dhurjati, P., Geoghegan Jr, R. F., & Robnett, B. (1988). Kinetic modelling of hybridoma cell growth and immunoglobulin production in a large- scale suspension culture. *Biotechnology and bioengineering*, 32(8), 1067-1072.

Bröker, M., Ragg, H., & Karges, H. E. (1987). Expression of human antithrombin III in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe. Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression*, 908(3), 203-213.

Buchetics, M., Dragosits, M., Maurer, M., Rebnegger, C., Porro, D., Sauer, M., ... & Mattanovich, D. (2011). Reverse engineering of protein secretion by uncoupling of cell cycle phases from growth. *Biotechnology and bioengineering*, 108(10), 2403-2412.

Burgard, J., Valli, M., Graf, A. B., Gasser, B., & Mattanovich, D. (2017). Biomarkers allow detection of nutrient limitations and respective supplementation for elimination in *Pichia pastoris* fed-batch cultures. *Microbial cell factories*, 16(1), 117.

Canales, C., Altamirano, C., & Berrios, J. (2018). The growth of *Pichia pastoris* Mut+ on methanol–glycerol mixtures fits to interactive dual-limited kinetics: model development and application to optimized fed-batch operation for heterologous protein production. *Bioprocess and biosystems engineering*, 1-12.

Carnicer, M., Ten Pierick, A., van Dam, J., Heijnen, J. J., Albiol, J., van Gulik, W., & Ferrer, P. (2012). Quantitative metabolomics analysis of amino acid metabolism in recombinant *Pichia pastoris* under different oxygen availability conditions. *Microbial cell factories*, 11(1), 83.

Çelik, E., Çalık, P., & Oliver, S. G. (2009). Fed- batch methanol feeding strategy for recombinant protein production by *Pichia pastoris* in the presence of co- substrate sorbitol. *Yeast*, 26(9), 473-484.

Cereghino, J. L., & Cregg, J. M. (2000). Heterologous protein expression in the methylotrophic yeast Pichia pastoris. FEMS microbiology reviews, 24(1), 45-66.

Charoenrat, T., Ketudat-Cairns, M., Jahic, M., Veide, A., & Enfors, S. O. (2006). Increased total air pressure versus oxygen limitation for enhanced oxygen transfer and product formation in a *Pichia pastoris* recombinant protein process. *Biochemical engineering journal*, 30(2), 205-211.

Chen, L., Mohsin, A., Chu, J., Zhuang, Y., Liu, Y., & Guo, M. (2017). Enhanced protein production by sorbitol co-feeding with methanol in recombinant *Pichia pastoris* strains. *Biotechnology and Bioprocess Engineering*, 22(6), 767-773.

Chongchittapiban, P., Borg, J., Waiprib, Y., Pimsamarn, J., & Tongta, A. (2016). On-line methanol sensor system development for recombinant human serum albumin production by *Pichia pastoris*. *African Journal of Biotechnology*, 15(42), 2374-2383.

Christen, S., & Sauer, U. (2011). Intracellular characterization of aerobic glucose metabolism in seven yeast species by 13C flux analysis and metabolomics. *FEMS yeast research*, 11(3), 263-272.

Clare, J. J., Rayment, F. B., Ballantine, S. P., Sreekrishna, K., & Romanos, M. A. (1991). High-level expression of tetanus toxin fragment C in *Pichia pastoris* strains containing multiple tandem integrations of the gene. *Bio/technology*, 9(5), 455.

Cos, O., Ramón, R., Montesinos, J. L., & Valero, F. (2006). Operational strategies, monitoring and control of heterologous protein production in the methylotrophic yeast *Pichia pastoris* under different promoters: a review. *Microbial cell factories*, 5(1), 17.

Couderc, R., & Baratti, J. (1980). Oxidation of methanol by the yeast, *Pichia pastoris*. Purification and properties of the alcohol oxidase. *Agricultural and biological chemistry*, 44(10), 2279-2289.

Emenike, V. N., Schenkendorf, R., & Krewer, U. (2018). Model-based optimization of biopharmaceutical manufacturing in *Pichia pastoris* based on dynamic flux balance analysis. *Computers & Chemical Engineering*, 118, 1-13.

Farias, D., de Andrade, R. R., & Maugeri-Filho, F. (2014). Kinetic modeling of ethanol production by *Scheffersomyces stipitis* from xylose. *Applied biochemistry and biotechnology*, 172(1), 361-379.

Ferreira, A. R., Ataíde, F., Von Stosch, M., Dias, J. M. L., Clemente, J. J., Cunha, A. E., & Oliveira, R. (2012). Application of adaptive DO-stat feeding control to *Pichia pastoris* X33 cultures expressing a single chain antibody fragment (scFv). *Bioprocess and biosystems engineering*, 35(9), 1603-1614.

Fleer, R., Yeh, P., Amellal, N., Maury, I., Fournier, A., Bacchetta, F., ... & Fukuhara, H. (1991). Stable multicopy vectors for high–level secretion of recombinant human serum albumin by *Kluyveromyces* Yeasts. *Bio/technology*, 9(10), 968.

Frame, K. K., & Hu, W. S. (1991). Kinetic study of hybridoma cell growth in continuous culture. I. A model for non- producing cells. *Biotechnology and bioengineering*, 37(1), 55-64.

Goldfeld, M., Christensen, J., Pollard, D., Gibson, E. R., Olesberg, J. T., Koerperick, E. J., ... & Evans, C. E. (2014). Advanced near- infrared monitor for stable real- time measurement and control of *Pichia pastoris* bioprocesses. *Biotechnology Progress*, 30(3), 749-759.

Gmeiner, C., Saadati, A., Maresch, D., Krasteva, S., Frank, M., Altmann, F., ... & Spadiut, O. (2015). Development of a fed-batch process for a recombinant *Pichia pastoris* Δ*och1* strain expressing a plant peroxidase. *Microbial cell factories*, 14(1), 1.

Güneş, H., Boy, E., Ata, Ö., Zerze, G. H., Çalık, P., & Özdamar, T. H. (2016). Methanol feeding strategy design enhances recombinant human growth hormone production *by Pichia pastoris. Journal of Chemical Technology & Biotechnology*, 91(3), 664-671.

Gurramkonda, C., Adnan, A., Gäbel, T., Lünsdorf, H., Ross, A., Nemani, S. K., ... & Rinas, U. (2009). Simple high-cell density fed-batch technique for high-level recombinant protein production with *Pichia pastoris*: Application to intracellular production of Hepatitis B surface antigen. *Microbial cell factories*, 8(1), 13.

Gurramkonda, C., Polez, S., Skoko, N., Adnan, A., Gäbel, T., Chugh, D., ... & Rinas, U. (2010). Application of simple fed-batch technique to high-level secretory production of insulin precursor using *Pichia pastoris* with subsequent purification and conversion to human insulin. *Microbial cell factories*, 9(1), 31.

Hartner, F. S., & Glieder, A. (2006). Regulation of methanol utilization pathway genes in yeasts. *Microbial Cell Factories*, 5(1), 39.

Heyland, J., Fu, J., Blank, L. M., & Schmid, A. (2010). Quantitative physiology of *Pichia pastoris* during glucose- limited high- cell density fed- batch cultivation for recombinant protein production. *Biotechnology and bioengineering*, 107(2), 357-368.

Heyland, J., Fu, J., Blank, L. M., & Schmid, A. (2011). Carbon metabolism limits recombinant protein production in *Pichia pastoris*. Biotechnology and Bioengineering, 108(8), 1942-1953.

Higgins, D. R. (2007). *Pichia* protocols (Vol. 268). J. M. Cregg (Ed.). Totowa: Humana Press.

Hou, W., Meng, X., Wang, Y., Mo, W., Wu, Y., & Yu, M. (2017). Characterization and high- yield production of non- N- glycosylated recombinant human BCMA- Fc in *Pichia pastoris*. *Engineering in Life Sciences*, 17(2), 96-106.

Irani, Z. A., Kerkhoven, E. J., Shojaosadati, S. A., & Nielsen, J. (2016). Genome- scale metabolic model of *Pichia pastoris* with native and humanized glycosylation of recombinant proteins. *Biotechnology and bioengineering*, 113(5), 961-969.

Invitrogen Corp. (2002). *Pichia Fermentation Process Guidelines*. Retrieved from: http://tools.thermofisher.com/content/sfs/manuals/pichiaferm_prot.pdf

Jahic, M., Wallberg, F., Bollok, M., Garcia, P., & Enfors, S. O. (2003). Temperature limited fed-batch technique for control of proteolysis in *Pichia pastoris* bioreactor cultures. *Microbial cell factories*, 2(1), 6.

Jin, H., Liu, G., Ye, X., Duan, Z., Li, Z., & Shi, Z. (2010). Enhanced porcine interferon-α production by recombinant *Pichia pastoris* with a combinational control strategy of low induction temperature and high dissolved oxygen concentration. *Biochemical Engineering Journal*, 52(1), 91-98.

Kim, S. J., Ha, G. S., Lee, G., Lim, S. I., Lee, C. M., Yang, Y. H., ... & Kim, C. W. (2018). Enhanced expression of soluble antibody fragments by low-temperature and overdosing with a nitrogen source. *Enzyme and microbial technology*, 115, 9-15.

Kurtzman, C. P. (2009). Biotechnological strains of *Komagataella (Pichia) pastoris* are *Komagataella phaffii* as determined from multigene sequence analysis. *Journal of industrial microbiology & biotechnology*, 36(11), 1435.

Lakowitz, A., Godard, T., Biedendieck, R., & Krull, R. (2018). Mini review: Recombinant production of tailored bio-pharmaceuticals in different *Bacillus strains* and future perspectives. *European Journal of Pharmaceutics and Biopharmaceutics*, 126, 27-39.

Levenspiel, O. (1980). The Monod equation: a revisit and a generalization to product inhibition situations. *Biotechnology and bioengineering*, 22(8), 1671-1687.

Li, L., Huang, C., Zhao, F., Deng, T., Lin, Y., Zheng, S., ... & Han, S. (2018). Improved production and characterization of *Volvariella volvacea* Endoglucanase 1 expressed in *Pichia pastoris. Protein expression and purification*, 152, 107-113.

Liu, M., Liang, Y., Zhang, H., Wu, G., Wang, L., Qian, H., & Qi, X. (2018). Production of a recombinant carrot antifreeze protein by *Pichia pastoris* GS115 and its cryoprotective effects on frozen dough properties and bread quality. *LWT*, 96, 543-550.

Looser, V., Bruhlmann, B., Bumbak, F., Stenger, C., Costa, M., Camattari, A., ... & Kovar, K. (2015). Cultivation strategies to enhance productivity of *Pichia pastoris*: a review. *Biotechnology advances*, 33(6), 1177-1193.

Luong, J. H. T. (1985). Kinetics of ethanol inhibition in alcohol fermentation. *Biotechnology and bioengineering*, 27(3), 280-285.

Mayer, A. F., Hellmuth, K., Schlieker, H., Lopez- Ulibarri, R., Oertel, S., Dahlems, U., ... & Van Loon, A. P. G. M. (1999). An expression system matures: A highly efficient and cost-effective process for phytase production by recombinant strains of *Hansenula polymorpha*. *Biotechnology and bioengineering*, 63(3), 373-381.

Oliveira, R., Clemente, J. J., Cunha, A. E., & Carrondo, M. J. T. (2005). Adaptive dissolved oxygen control through the glycerol feeding in a recombinant *Pichia pastoris* cultivation in conditions of oxygen transfer limitation. *Journal of biotechnology*, 116(1), 35-50.

Parashar, D., & Satyanarayana, T. (2016). Enhancing the production of recombinant acidic α-amylase and phytase in *Pichia pastoris* under dual promoters [constitutive (GAP) and inducible (AOX)] in mixed fed batch high cell density cultivation. Process biochemistry, 51(10), 1315-1322.

Park, E. H., Shin, Y. M., Lim, Y. Y., Kwon, T. H., Kim, D. H., & Yang, M. S. (2000). Expression of glucose oxidase by using recombinant yeast. *Journal of Biotechnology*, 81(1), 35-44.

Peebo, K., & Neubauer, P. (2018). Application of Continuous Culture Methods to Recombinant Protein Production in Microorganisms. *Microorganisms*, 6(3), 56.

Pichia Produced Products on the Market. (n.d.). Retrieved November 16, 2018, from https://pichia.com/science-center/commercialized-products/

Picotto, L. D., Sguazza, G. H., Tizzano, M. A., Galosi, C. M., Cavalitto, S. F., & Pecoraro, M. R. (2017). An effective and simplified DO-stat control strategy for production of rabies glycoprotein in *Pichia pastoris*. *Protein expression and purification*, 132, 124-130.

Pimentel, N., Rodríguez- Lopez, A., Díaz, S., Losada, J. C., Díaz- Rincón, D. J., Cardona, C., ... & Poutou- Piñales, R. A. (2018). Production and characterization of a human lysosomal recombinant iduronate- 2- sulfatase produced in *Pichia pastoris*. Biotechnology and applied biochemistry, 65(5), 655-664.

Ponte, X., Montesinos-Seguí, J. L., & Valero, F. (2016). Bioprocess efficiency in *Rhizopus oryzae* lipase production by *Pichia pastoris* under the control of PAOX1 is oxygen tension dependent. *Process Biochemistry*, 51(12), 1954-1963.

Ponte, X., Barrigón, J. M., Maurer, M., Mattanovich, D., Valero, F., & Montesinos- Seguí, J. L. (2018) Towards optimal substrate feeding for heterologous protein production in *Pichia pastoris (Komagataella spp*) fed- batch processes under P AOX1 control: a modeling aided approach. *Journal of Chemical Technology & Biotechnology*.

Porro, D., Sauer, M., Branduardi, P., & Mattanovich, D. (2005). Recombinant protein production in yeasts. Molecular biotechnology, 31(3), 245-259.

Potgieter, T. I., Kersey, S. D., Mallem, M. R., Nylen, A. C., & d'Anjou, M. (2010). Antibody expression kinetics in glycoengineered *Pichia pastoris*. *Biotechnology and Bioengineering*, 106(6), 918-927.

Potvin, G., Ahmad, A., & Zhang, Z. (2012). Bioprocess engineering aspects of heterologous protein production in *Pichia pastoris*: a review. *Biochemical Engineering Journal*, 64, 91-105.

Presland, J., & Strange, P. G. (1998). Pharmacological characterization of the D2 dopamine receptor expressed in the yeast *Schizosaccharomyces pombe*. *Biochemical pharmacology*, 56(5), 577-582.

Prielhofer, R., Cartwright, S. P., Graf, A. B., Valli, M., Bill, R. M., Mattanovich, D., & Gasser, B. (2015). *Pichia pastoris* regulates its gene-specific response to different carbon sources at the transcriptional, rather than the translational, level. *BMC genomics*, 16(1), 167.

Prielhofer, R., Reichinger, M., Wagner, N., Claes, K., Kiziak, C., Gasser, B., & Mattanovich, D. (2018). Superior protein titers in half the fermentation time: Promoter and process engineering for the glucose- regulated GTH1 promoter of *Pichia pastoris*. *Biotechnology and bioengineering*.

Ramsay, A. M., & Douglas, L. J. (1979). Effects of phosphate limitation of growth on the cell-wall and lipid composition of *Saccharomyces cerevisiae. Microbiology*, 110(1), 185-191.

Systems-level organization of yeast methylotrophic lifestyle. BMC biology, 13(1), 80.

Rebnegger, C., Graf, A. B., Valli, M., Steiger, M. G., Gasser, B., Maurer, M., & Mattanovich, D. (2014). In *Pichia pastoris*, growth rate regulates protein synthesis and secretion, mating and stress response. *Biotechnology journal*, 9(4), 511-525.

Rocha, T. L., Paterson, G., Crimmins, K., Alan, B. O. Y. D., Sawyer, L., & Fothergill-Gilmore, L. A. (1996). Expression and secretion of recombinant ovine β-lactoglobulin in *Saccharomyces cerevisiae* and *Kluyveromyces lactis*. *Biochemical Journal*, 313(3), 927-932.

Romanos, M. A., Makoff, A. J., Fairweather, N. F., Beesley, K. M., Slater, D. E., Rayment, F. B., ... & Clare, J. J. (1991). Expression of tetanus toxin fragment C in yeast: gene synthesis is required to eliminate fortuitous polyadenylation sites in AT-rich DNA. *Nucleic acids research*, 19(7), 1461-1467.

Rußmayer, H., Buchetics, M., Gruber, C., Valli, M., Grillitsch, K., Modarres, G., ... & Steiger, M. (2015).

Rumjantsev, A. M., Bondareva, O. V., Padkina, M. V., & Sambuk, E. V. (2014). Effect of nitrogen source and inorganic phosphate concentration on methanol utilization and PEX genes expression in *Pichia pastoris*. *The Scientific World Journal*, 2014.

Safder, I., Khan, S., Islam, I. U., & Kazim, M. (2018). *Pichia pastoris* expression system: a potential candidate to express protein in industrial and biopharmaceutical domains. *Biomedical Letters*, 4(1), 1-13.

Sander, P., Grünewald, S., Reiländer, H., & Michel, H. (1994). Expression of the human D2S dopamine receptor in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*: a comparative study. *FEBS letters*, 344(1), 41-46.

Schröder M, Chang J, Kaufman R. The unfolded protein response represses nitrogen-starvation induced developmental differentiation in yeast

Şeker, Ş., Beyenal, H., Salih, B., & Tanyolac, A. (1997). Multi-substrate growth kinetics of *Pseudomonas putida* for phenol removal. *Applied Microbiology and Biotechnology*, 47(5), 610-614.

Singh, A. B., Sharma, A. K., & Mukherjee, K. J. (2012). Analyzing the metabolic stress response of recombinant *Escherichia coli* cultures expressing human interferon-beta in high cell density fed batch cultures using time course transcriptomic data. *Molecular BioSystems*, 8(2), 615-628.

Singh, A., & Narang, A. (2019). The Mut+ strain of Komagataella phaffii (Pichia pastoris) expresses PAOX1 5 and 10 times faster than Muts and Mut-strains: Evidence that formaldehyde or/and formate are true inducers of AOX. bioRxiv, 573519.

Sleep, D., Belfield, G. P., Ballance, D. J., Steven, J., Jones, S., Evans, L. R., ... & Goodey, A. R. (1991). *Saccharomyces cerevisiae* strains that overexpress heterologous proteins. *Bio/technology*, 9(2), 183.

Song, Liang, Yuan, & Song, L. (2007). Retarded Transport and Accumulation of Soluble Microbial Products in a Membrane Bioreactor. *Journal of Environmental Engineering*, 133(1), 36-43.

Sohn, J. H., Choi, E. S., Chung, B. H., Youn, D. J., Seo, J. H., & Rhee, S. K. (1995). Process development for the production of recombinant hirudin in *Saccharomyces cerevisiae*: from upstream to downstream. *Process biochemistry*, 30(7), 653-660.

Sohn, S. B., Graf, A. B., Kim, T. Y., Gasser, B., Maurer, M., Ferrer, P., ... & Lee, S. Y. (2010). Genome- scale metabolic model of methylotrophic yeast *Pichia pastoris* and its use for in silico analysis of heterologous protein production. *Biotechnology journal*, 5(7), 705-715.

Sun, W., Lai, Y., Li, H., Nie, T., Kuang, Y., Tang, X., ... & Wu, D. (2016). High level expression and purification of active recombinant human interleukin-15 in *Pichia pastoris. Journal of immunological methods*, 428, 50-57.

Tervasmäki, P., Latva-Kokko, M., Taskila, S., & Tanskanen, J. (2018). Effect of oxygen transfer on yeast growth—Growth kinetic and reactor model to estimate scale-up effects in bioreactors. *Food and Bioproducts Processing*, 111, 129-140.

Theron, C. W., Berrios, J., Delvigne, F., & Fickers, P. (2018). Integrating metabolic modeling and population heterogeneity analysis into optimizing recombinant protein production by *Komagataella (Pichia) pastoris. Applied microbiology and biotechnology*, 102(1), 63-80.

Tøttrup, H. V., & Carlsen, S. (1990). A process for the production of human proinsulin in *Saccharomyces cerevisiae. Biotechnology and bioengineering*, 35(4), 339-348.

Villadsen, J., Nielsen, J., & Lidén, G. (2011). Growth Kinetics of Cell Cultures. In *Bioreaction Engineering Principles* (pp. 271-357). Springer, Boston, MA.

Vos, T., Hakkaart, X. D., de Hulster, E. A., van Maris, A. J., Pronk, J. T., & Daran-Lapujade, P. (2016). Maintenance-energy requirements and robustness of *Saccharomyces cerevisiae* at aerobic near-zero specific growth rates. *Microbial cell factories*, 15(1), 111.

Wang, X., Sun, Y., Ke, F., Zhao, H., Liu, T., Xu, L., ... & Yan, Y. (2012). Constitutive expression of *Yarrowia lipolytica* lipase LIP2 in *Pichia pastoris* using GAP as promoter. *Applied biochemistry and biotechnology*, 166(5), 1355-1367.

Weydemann, U., Keup, P., Piontek, M., Strasser, A. W. M., Schweden, J., Gellissen, G., & Janowicz, Z. A. (1995). High-level secretion of hirudin by *Hansenula polymorpha*—authentic processing of three different preprohirudins. *Applied microbiology and biotechnology*, 44(3-4), 377-385.

Yamada, Matsuda, Maeda, & Mikata. (1995). The phylogenetic relationships of methanol-assimilating yeasts based on the partial sequences of 18S and 26S ribosomal RNAs: The proposal of *Komagataella* gen. nov. (*Saccharomycetaceae*). *Bioscience, Biotechnology, and Biochemistry,* 59(3), 439-44.

Ye, J., Ly, J., Watts, K., Hsu, A., Walker, A., McLaughlin, K., ... & Pollard, D. (2011). Optimization of a glycoengineered *Pichia pastoris* cultivation process for commercial antibody production. *Biotechnology progress*, 27(6), 1744-1750.

Yu, X. W., Sun, W. H., Wang, Y. Z., & Xu, Y. (2017). Identification of novel factors enhancing recombinant protein production in multi-copy *Komagataella phaffii* based on transcriptomic analysis of overexpression effects. *Scientific reports*, 7(1), 16249.

Zhang, M., Yu, X. W., Xu, Y., Jouhten, P., Swapna, G. V., Glaser, R. W., ... & Szyperski, T. (2017). 13C metabolic flux profiling of *Pichia pastoris* grown in aerobic batch cultures on glucose revealed high relative anabolic use of TCA cycle and limited incorporation of provided precursors of branched- chain amino acids. *The FEBS journal*, 284(18), 3100-3113.

Zhang, W., Bevins, M., Plantz, B., Smith, L., & Meagher, M. (2000). Modeling Pichia pastoris growth on methanol and optimizing the production of a recombinant protein, the heavy-chain fragment C of botulinum neurotoxin, serotype A. Biotechnology and Bioengineering, 70(1), 1-8.

Zhang, W., Sinha, J., Smith, L. A., Inan, M., & Meagher, M. M. (2005). Maximization of production of secreted recombinant proteins in *Pichia pastoris* fed- batch fermentation. *Biotechnology progress*, 21(2), 386-393.

APPENDIX A: SUPPLEMENTARY INFORMATION (COMPUTER CODE)

Equations used in model generation:

Mass Balance around biomass**:**

$$\frac{d(X \cdot V)}{dt} = \mu \cdot V \cdot X$$

Mass Balance around substrate**:**

$$\frac{d(S \cdot V)}{dt} = F(t) \cdot S_o - (\frac{\mu}{Y_{XS}} + m) \cdot V \cdot X$$

Change in volume due to feeding**:**

$$\frac{dV}{dt} = F(t)$$

General Monod Equation for growth**:**

$$\mu = \frac{\mu_{max} \cdot S}{K_s + S}$$

Equations for various feeding methods (linear –or– exponential feeding with maintenance):

$$F(t) = a \cdot t + b \quad -or- \quad F(t) = a \cdot e^{d \cdot t + m}$$

Equations inputted in ODE assistant module:

diff(S(t),t)*V(t)+S(t)*diff(V(t),t) = F(t)*So-(u(t)/Yxs+m)*V(t)*X(t)

diff(V(t),t) = F(t)

diff(X(t),t)*V(t)+X(t)*diff(V(t),t) = u(t)*V(t)*X(t)

F(t) = a*t+b+c*e^(d*t+f*m)

u(t) = umax*S(t)/(Ks+S(t))

Raw code for constant feed trends:

```
sol1 := dsolve([diff(S(t),t)*V(t)+S(t)*diff(V(t),t) = 700*F(t)-(1.97005516154452*u(t)+.8e-2)*V(t)*X(t), diff(V(t),t) = F(t), diff(X(t),t)*V(t)+X(t)*diff(V(t),t) = u(t)*V(t)*X(t), F(t) = .283e-1, u(t) = .26*S(t)/(.2+S(t)), S(0) = .1e-1, V(0) = 2.5, X(0) = 75], numeric);
plots[odeplot](sol1, 0..10, color = red, view = [DEFAULT, 0 .. .1]);
```

Raw code for linear feed trends:

```
sol1 := dsolve([diff(S(t),t)*V(t)+S(t)*diff(V(t),t) = 700*F(t)-(1.97005516154452*u(t)+.8e-2)*V(t)*X(t), diff(V(t),t) = F(t), diff(X(t),t)*V(t)+X(t)*diff(V(t),t) = u(t)*V(t)*X(t), F(t) = .5e-2*t+.283e-1, u(t) = .260*S(t)/(.2+S(t)), S(0) = .1e-4, V(0) = 2.5, X(0) = 75], numeric);
plots[odeplot](sol1, [t, u(t)], 0..50, color = red, view = [DEFAULT, 0 .. .1]);
```

Raw code for stepped increase feed trends:

```
sol1 := dsolve([diff(S(t),t)*V(t)+S(t)*diff(V(t),t) = 700*F(t)-(1.97005516154452*u(t)+.8e-2)*V(t)*X(t), diff(V(t),t) = F(t), diff(X(t),t)*V(t)+X(t)*diff(V(t),t) = u(t)*V(t)*X(t), F(t) = piecewise(t < 0,0,0 <= t and t < 5,.2e-1,5 <= t and t < 10,.4e-1,10 <= t and t < 15,.6e-1,15 <= t and t < 20,.8e-1,20 <= t and t < 25,.1,25 <= t and t < 30,.12,30 <= t and t < 35,.14,35 <= t and t < 40,.16,40 <= t and t < 45,.18,45 <= t,.2), u(t) = .26*S(t)/(.2+S(t)), S(0) = .1e-4, V(0) = 2.5, X(0) = 75], numeric, method = rosenbrock_dae);
plots[odeplot](sol1, [t, u(t)], 0..50, color = red, view = [DEFAULT, 0 .. .1]);
```

Raw code for Timed Pulse feed trends:

```
sol1 := dsolve([(diff(S(t), t))*V(t)+S(t)*(diff(V(t), t)) = 700*F(t)-(1.97005516154452*u(t)+0.8e-2)*V(t)*X(t), diff(V(t), t) = F(t), (diff(X(t), t))*V(t)+X(t)*(diff(V(t), t)) = u(t)*V(t)*X(t), F(t) = piecewise(t < 0, 0, 0 <= t and t < 1, 1, 1 <= t and t < 5, 0, 5 <= t and t < 6, 1, 6 <= t and t < 10, 0, 10 <= t and t < 11, 1, 11 <= t and t < 15, 0, 15 <= t and t < 16, 1, 16 <= t and t < 20, 0, 20 <= t and t < 21, 1, 21 <= t and t < 25, 0, 25 <= t and t < 26, 1, 26 <= t and t < 30, 0, 30 <= t and t < 31, 1, 31 <= t and t < 35, 0, 35 <= t and t < 36, 1, 36 <= t and t < 40, 0, 40 <= t and t < 41, 1, 41 <= t and t < 45, 0, 45 <= t and t < 46, 1, 46 <= t, 0), u(t) = .26*S(t)/(.2+S(t)), S(0) = 0.1e-4, V(0) = 2.5, X(0) = 75], numeric, maxfun = 10000000); sol1(0.); plots[odeplot](sol1, [t, X(t)], 0 .. 50, color = red, view = [DEFAULT, 0 .. 300])
```

Raw code for exponential feed trends:

```
sol1 := dsolve([diff(S(t),t)*V(t)+S(t)*diff(V(t),t) = 700*F(t)-(1.97005516154452*u(t)+.8e-2)*V(t)*X(t), diff(V(t),t) = F(t), diff(X(t),t)*V(t)+X(t)*diff(V(t),t) = u(t)*V(t)*X(t), F(t) = .283e-1*2.71828182^(.5e-1*t+.8e-2), u(t) = .26*S(t)/(.2+S(t)), S(0) = .1e-4, V(0) = 2.5, X(0) = 75], numeric);
plots[odeplot](sol1, [t, X(t)], 0..50, color = red, view = [DEFAULT, 0 .. 300]);
```