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Macroscopic modelling of the growth and substrate consumption of wild type and genetically modified *Pichia pastoris*

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Abstract

Pichia pastoris is a popular yeast platform to generate several industrially relevant products which have applications in a wide range of sectors. The complexities in the processes due to the addition of a foreign gene are not widely explored. Since these complexities can be dependent on the strain characteristics, promoter, and type of protein produced, it is vital to investigate the growth and substrate consumption patterns of the host to facilitate customised process optimisation. In this study, the growth rates of *P. pastoris* GS115 wild type and genetically modified strains grown on glycerol and methanol in batch cultivation mode were estimated and the model providing the best representation of the true growth kinetics based on substrate consumption was identified. It was observed that the growth of *P. pastoris* exhibits Haldane kinetics on glycerol rather than the most commonly used Monod kinetics due to the inability of the latter to describe growth inhibition at high concentrations of glycerol. Whereas, the Cardinal Parameter Model, a newly proposed model for this application, was found to be the best fitting to describe the growth of *P. pastoris* on methanol due to its ability to describe methanol toxicity. Interestingly, the findings from this study concluded that in both substrates, the genetically engineered strain exhibited a higher growth rate compared to the wild type strain. Such an observation has not been established yet in other published works, indicating an opportunity to further optimise the carbon source feeding strategies when the host is grown in fed-batch mode.

Keywords: *Pichia pastoris, bioprocess, macroscopic modelling, substrate consumption*

Abbreviations

AOX: Alcohol Oxidase; Mut: Methanol utilisation; WT: Wild Type; GM: Genetically Modified; BSM: Basal Salt Medium; YPD: Yeast Peptone Dextrose; HPLC: High Performance Liquid Chromatography; GC: Gas Chromatography; SALLE: Salting Assisted Liquid-Liquid Extraction; CFU: Colony Forming Unit; CPM: Cardinal Parameter Model; WMSE: Weighted Mean Squared Error

1 Introduction

In the production of bio-based products, microorganisms play a crucial role. Among eukaryotes, *Pichia pastoris* has proven to be an ideal host to produce recombinant proteins. So far, *P. pastoris* has yielded almost 5000 recombinant proteins (Wang et al., 2021; Cereghino & Cregg, 2000), and current studies are targeted to better understand the cellular behaviour of the organism. The capacity to grow on low-cost media, ease of genetic manipulation and the ability for post-translational modification are some of the reasons for the prominence of this host in bioprocesses (Joseph et al., 2019). It possesses a methanol-regulated alcohol oxidase (AOX1) promoter that is widely utilised for recombinant protein production, and it can grow to high cell densities (Digan et al., 1989). Moreover, it can produce recombinant proteins both intracellularly and extracellularly (Brierley et al., 1990, Clare et al., 1991, Duff and Murray, 1988). Furthermore, due to its ability to secrete recombinant products in the milligram-to-gram range, it is a promising organism for both laboratory studies and industrial production (Hellwig et al. 2001).

Optimal bioprocessing conditions have to be determined for culturing the microbial host and producing recombinant proteins. The productivity of the bioprocess may be affected by strain engineering, the recombinant protein in question, and process conditions. Understanding the relationship between the bioprocessing conditions, microbial growth, and production behaviour of the host can lead to optimisation of the feeding profile in terms of (i) growth to high cell densities and (ii) production yield of the recombinant protein. The majority of *P. pastoris* bioprocesses are fed-batch processes, which have the benefit of maximising cell densities and thereby, contributing to maximum protein production. Typically, *P. pastoris* fermentations involve a three-phase feeding strategy starting with a glycerol batch phase, followed by a glycerol fed-batch phase, and finally inducing the expression using a methanol fed-batch phase. Previous studies (Shioya, 1992; Chen et al., 1997; D'Anjou & Daugulis, 2001)

have shown that controlling substrate feeding improves the productivity of the microorganism for the production of recombinant products. Therefore, it is vital to understand the metabolic behaviour of the host organism concerning the substrates used for cultivation to alleviate the cellular stresses that can reduce the growth and gene expression. Furthermore, understanding the growth rate of the organism is critical because it can influence the overall recombinant protein production. For metabolic activities, *P. pastoris* can use a variety of carbon sources, the most common of which are glucose, glycerol, and methanol.

Nowadays, glycerol has been favoured as a main carbon source for the cultivation of microorganisms. It offers a cheaper alternative to glucose due to the increased availability of glycerol attained from biodiesel production, making it more economical (Yazdani and Gonzalez, 2007, Khanal et al., 2008, Rausch and Belyea, 2006). The replacement is also advantageous because glycerol is seen to improve cell viability and recombinant protein productivity. Glycerol displays a higher degree of reduction per carbon compared to other sugars, which aid in higher yields of products (Clomburg & Gonzalez, 2013).

Yeasts can use methanol as an expression inducer and carbon source. A key enzyme in *P. pastoris* engaged in the dissimilation of methanol is alcohol oxidase (AOX) and the most used promoter for heterologous protein production is AOX1. There are two ways the AOX1 gene is regulated, i.e., (i) a repression/derepression and (ii) an induction mechanism (Tschopp et al., 1987, Ozimek et al., 2005). The former takes place in the presence of carbon sources like glucose or ethanol (Cereghino and Cregg, 2000, Cregg et al., 1989, Cregg et al., 2000) and for the latter, methanol is imperative. *P. pastoris* is capable of assimilating methanol at low concentrations. High methanol levels can cause toxicity to the cells as formaldehyde and hydrogen peroxide, two oxidised products of methanol, start accumulating (Couderc and Baratti, 1980; Cregg and Madden, 1988; Van der Klei et al., 1990). A fed-batch operation is therefore recommended for *P. pastoris* cultivation to maintain low methanol levels in the

culture medium capable of supporting high cell density formation. However, the identification of the optimal methanol concentration for *P. pastoris* fermentation is protein specific (Liu et al., 2020). Therefore, it is crucial to find the methanol concentration range in which the host organism can thrive and produce sufficiently high quantities of proteins since elevated levels of methanol have an inhibitory effect on cell growth.

Modelling the growth of *P. pastoris* cultivated in the bioreactors can help to comprehend the cellular dynamics of the organism. Furthermore, it can play a pivotal role in the optimisation of bioreactor operations. The complexity of the constructed model depends on the extent of information included. For instance, genome-scale stoichiometric models (Feist et al., 2007, Sohn et al., 2010) incorporate cellular interactions that can provide an extensive understanding of the bioprocesses. However, simple macroscopic models offer the advantage that they can be fine-tuned to a specific process relatively easily through parameter estimation and that they are suitable for model predictive control (Craven et al., 2014; Ramaswamy et al., 2005). Therefore, elaboration of a simple model is preferred only when required (Villadsen et al., 2011, Levenspiel, 2002). For *P. pastoris*, however, limited macroscopic models have been published so far (Barrigon et al., 2015; Çelik et al., 2009; Jahic et al., 2002; Ren et al., 2003). These works have focussed on specific bioproductions. However, it is unclear to what extent the growth and consumption behaviour of *P. pastoris* depends on the specific genetic modification. Assessing the growth of the organism in presence of different substrate concentrations in a fed-batch operation can be a tedious task and often generates results that are difficult to interpret. Therefore, to study how the host system responds to different concentrations of glycerol and methanol in a fed-batch process, the construction of macroscopic models describing kinetics in the batch mode can be beneficial.

The goal of this study is to investigate if the growth and substrate consumption of *P. pastoris* can be captured in generalised dynamic models that are applicable for simulating and

optimising various recombinant protein production processes. To this end, a case study is used where dynamic models are constructed for both a wild type GS115 Mut^+ and a genetically modified strain producing the recombinant thaumatin II protein. The production of thaumatin, a promising natural sweetener that can potentially replace sugar and artificial sweeteners is expected to create market opportunities in the forthcoming years. Since the commercial production of this plant-derived natural sweetener has constraints in terms of raw material availability, taste quality, and cost-effectiveness, a biotechnological production approach is required to overcome these drawbacks. Hence, in this study, the production of recombinant thaumatin is considered as a case study to understand how the growth and substrate consumption pattern of a genetically engineered *P. pastoris* differs from a wild type strain. The models developed in this study describe the growth and substrate consumption behaviour on both glycerol and methanol. To the author's knowledge, this is the first study comparing the growth and substrate consumption of a *P. pastoris* wild type and genetically modified strain.

2 Materials and methods

2.1 Experimental setup

Pichia pastoris GS115

In this study, *Pichia pastoris* GS115 His^+, a methanol utilization positive (Mut^+) phenotype wild type (WT) and a genetically modified (GM) strain with a subcloned vector pPICZalpfaB capable of expressing thaumatin II gene consisting of the pre-pro sequence was used. Genetic modification of the *P. pastoris* GS115 host was performed in VIB Protein Core (Zwijnaarde) by cloning pre-thaumatin-II-pro gene into the GS115 wild type strain. The strains were stored in glycerol stocks (5% (w/v) Yeast Peptone Dextrose (YPD, Carl Roth, Germany) broth, and 25% (w/v) glycerol (99+%, Chem Lab, Germany)) at -80 °C until further use. Preculture plates containing YPD medium (5% (w/v)) and bacteriological agar (1.6% (w/v))

(VWR, Belgium) were used for both GS115 WT and GS115 GM strains and incubated at 30 °C for three days. The first preculture was prepared by inoculating 20 mL YPD (yeast extract-peptone-dextrose) medium in a 100 mL Erlenmeyer flask using three colonies from the YPD agar plates. After 24 h on a rotary shaker (VELP Scientifica Srl, Italy) at 220 rpm, 20 µL of the first preculture was used to inoculate the second preculture of 80 mL YPD medium in a 250 mL Schott bottle. The second preculture was stored at 20 °C over the weekend on rotary shakers at 160 rpm. Prior to inoculation of the bioreactor medium, 0.5 mL of the preculture was diluted with 4.5 mL of 0.85% sodium chloride (Sigma-Aldrich, Germany) solution. The 5 mL preculture was introduced into the bioreactor using a sterile 10 mL syringe (BD DiscarditTMII, USA) and needle through the injection port to attain an initial cell concentration of 10 ln (CFU/mL).

Cultivation medium

For the bioreactor experiments, basal salt medium (BSM) was used. This medium contained 26.7 g phosphoric acid (H_3PO_4), 0.93 g calcium sulfate ($CaSO_4$), 18.2 g potassium sulfate (K_2SO_4), 14.87 magnesium sulfate heptahydrate ($MgSO_4(H_2O)_7$), 4.13 g potassium hydroxide (KOH). Following sterilization of the media, 4 mL pichia trace salts (PTM1) and 4 mL biotin were added into the reactor aseptically using a sterile 10 mL syringe and needle. The components of the PTM1 trace salts solution included 6.0 g copper sulphate ($CuSO_4 \cdot 5H_2O$), 0.08 g sodium iodide (NaI), 3.0 g manganese (II) sulfate hydrate ($MnSO_4 \cdot H_2O$), 0.2 g sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$), 0.02 g boric acid (H_3BO_3), 0.5 g cobalt (II) chloride ($CoCl_2$), 20.0 g zinc chloride ($ZnCl_2$), 65.0 g ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), 0.2 g biotin and 5.0 mL of 96% sulfuric acid (H_2SO_4) in per liter.

Bioreactor batch operation

Pichia pastoris cultivations were performed in a 5 L benchtop bioreactor (Biostat B, Sartorius Stedim GmbH) in the batch mode. Prior to autoclaving, 1.5 L of BSM medium

prepared according to the "Pichia Fermentation Process Guidelines" (Invitrogen, San Diego, CA, U.S.A.) was added into the bioreactor. Trace salts containing Na^+, K^+, Mg^{2+}, Ca^{2+}, SO_4^{2-}, Cl, and PO_4^{3-} and biotin were added into the reactor post sterilization. The cultivation temperature was set at 30 °C using a temperature probe (Sartorius PT100) and pH was adjusted to 6.0 by adding 28% NH_4OH (Carl Roth) and monitored using a probe (Hamilton EasyFerm plus VP). The dissolved oxygen level was set at 40% using a cascade control system where an air flow rate between 0 to 5 L/min and agitation was provided between 500 and 800 rpm when the aeration rate reached the maximum. Antifoam (Y-30 emulsion) at 1:5 dilution was added into the media at specific time points to reduce foaming. For experiments using methanol, the substrate was introduced into the reactor using a peristaltic pump (model 7519-10, MasterFlex L/S, Cole-Parmer) prior to inoculation of the media. Monitoring and recording of the process variables was executed using the MFCS/win software (Sartorius Stedim GmbH). The initial concentrations studied for the batch experiments were as shown in Table 1. Substrate concentrations were iteratively selected based on the experimental and modelling results.

Table 1: Different concentrations of glycerol and methanol investigated in batch operation.

***P. pastoris* strain**	**Initial substrate concentration in the medium (g/L)**	
	Glycerol	**Methanol**
GS115 WT	10, 20, 30, 40, 70, 100	5, 10, 20, 30
GS115 GM	1, 5, 10, 20, 30, 40, 70, 100	2, 5, 10, 20, 30

Sampling and analytical measurements

The viable cell count was monitored every hour by a conventional plate count approach using YPD agar plates. For this, 100 µL of unfiltered biological sample was diluted in 900 µL of 0.85% (w/v) sodium chloride solution (Sigma-Aldrich, Germany). From the dilutions, three drops were transferred to YPD agar plates and incubated at 30 °C for three days.

The biological samples were filtered using 0.2 µm filters for the analytical measurements and stored at -80 °C until further processing. Glycerol quantification was performed using HPLC (Agilent 1260 Infinity II) equipped with an Aminex HPX-87H column (300 x 7.8 mm). The mobile phase used was 0.005 M H_2SO_4 at a flow rate of 0.6 mL/min at a column temperature of 50 °C. Sample pre-processing was performed to remove unwanted impurities by adding 10 µL of 5% (w/v) sodium deoxycholate solution and 100 µL of 100% trichloroacetic acid into 900 µL of the thawed sample. The sample was stored at -20 °C for 1 hour and centrifuged at 10,000 g for 15 mins. The supernatant was carefully transferred into an HPLC sample vial (Fischer Scientific, Poland) and injected immediately. The samples were injected three times and the results were expressed as their mean and standard deviation.

The methanol present in the samples was quantified using an Agilent 8860 gas chromatography system (Agilent Technologies, California, USA) equipped with a Flame Ionisation Detector (Agilent technologies) as detailed in Joseph et al. (2022). Prior to the analysis, the methanol present in the complex mixture was separated by using the salting assisted liquid-liquid extraction (SALLE) technique. SALLE was conducted by adding 0.772 g of potassium carbonate into 700 µL of filtered sample for salting out followed by 700 µL of ethyl acetate. The supernatant attained after spinning down the samples was transferred into GC sample vial (Fischer Scientific, Poland) prior to analysis. For the separation of the analyte, an HP-5 capillary column 30 m x 0.320 mm i.d. x 0.25 µm film thickness (Agilent Technologies, USA) was used. An ALS syringe (10 µL, fixed needle, 23-26s/42/cone, Agilent Technologies, USA) was used to inject 1 µL of the pre-processed sample in split/splitless mode depending on the concentration range of the analyte present in the sample. The detector and injector temperatures used were 300 and 225 °C respectively. The temperature program was initiated by holding at 40°C for 3 minutes and increasing to 60 °C/min to reach 225 °C. The GC data was recorded using OpenLab CDS software (version 2.4). Each sample was injected

three times. The raw data attained from the analysis was examined using the OpenLab CDS software (Agilent Technologies).

2.2 Bioprocess modelling

Model equations

A simple bioprocess model was constructed using two coupled differential equations for the growth of cells X [CFU/mL] and the consumption of substrate S [mg/mL], which was either glycerol or methanol:

$$\frac{dX(t)}{dt} = \mu\big(S(t)\big) \cdot X(t) \quad \text{with} \quad X(t=0) = X_0 \tag{1}$$

$$\frac{dS(t)}{dt} = -\frac{1}{Y_{X/S}} \cdot \mu\big(S(t)\big) \cdot X(t) \quad \text{with} \quad S(t=0) = S_0 \tag{2}$$

with μ the maximum specific growth rate [1/h], X_0 the initial cell density [CFU/mL], $Y_{X/S}$ the yield of cells per quantity of substrate [CFU/mg] and S_0 the initial substrate concentration [mg/mL]. Given the exponential increase of the cell population, cell density data was logarithmically transformed for parameter estimation purposes. Moreover, also the yield parameter $Y_{X/S}$ was estimated on a logarithmic scale. X_0 and S_0 are assumed to be unknown and are therefore estimated as separate model parameters for each experiment. Also $Y_{X/S}$ was estimated as a separate model parameter for each experiment to be able to observe any differences in the yield caused by carbon sources, yeast strains and substrate concentrations. A maintenance term was tested but was found to be inapplicable for the data that was obtained (results not shown). As such, this term was omitted from the bioprocess model. The rate of growth and substrate consumption is determined by a model that relates the maximum specific growth rate to the substrate concentration. For this purpose, three different models were tested in this research, i.e., the Monod equation, the Haldane equation and the Cardinal Parameter Model (CPM). The Monod equation assumes that the growth rate increases asymptotically to

its maximum with an increasing substrate concentration (Monod, 1949). This equation is written as:

$$\mu\big(S(t)\big) = \mu_{opt} \cdot \frac{S(t)}{S(t) + K_s} \tag{3}$$

with μ_{opt} the optimum growth rate [1/h] and K_s the half-saturation constant [mg/mL] at which half of the optimum growth rate is reached. The Haldane equation describes substrate inhibition both due to low and high levels of substrate, leading to the existence of an optimum substrate concentration at which the optimum growth rate is reached (Haldane, 1930). A modified formulation of this equation was used that ensures that the optimum growth rate is an interpretable parameter:

$$\mu\big(S(t)\big) = \mu_{opt} \cdot \frac{S(t)}{S(t) + K_m + \frac{S(t)^2}{K_i}} \cdot \frac{2 \cdot K_m + \sqrt{K_m \cdot K_i}}{\sqrt{K_m \cdot K_i}} \tag{4}$$

with K_m the Michaelis constant [mg/mL] and K_i the inhibition constant [mg/mL]. The third model, the CPM, was taken from the discipline of predictive microbiology where it originated in the work of Rosso et al. (1993). This model was constructed to describe the effect of environmental conditions such as temperature and pH on the maximum growth rate. This model has never before been used to describe the effect of substrate concentration on the microbial growth rate. It is formulated as:

$$\mu\big(S(t)\big) = \mu_{opt} \cdot \frac{(S(t) - S_{min}) \cdot (S(t) - S_{max})^k}{(S_{opt} - S_{max})^{(k-1)} \cdot \Big((S_{opt} - S_{max}) \cdot (S(t) - S_{opt}) - (S_{opt} - S_{min}) \cdot \big((k-1) \cdot S_{opt} + S_{max} - k \cdot S(t)\big)\Big)} \tag{5}$$

with S_{min} and S_{max} the minimum and maximum substrate concentrations that support growth [mg/mL], S_{opt} the optimum substrate concentration [mg/mL] at which the optimum growth rate is reached and k a shape parameter [-]. The growth rate is assumed to be zero for all substrate concentrations outside the range $[S_{min};\ S_{max}]$. Given that growth is assumed to occur

as long as any substrate is present, the value of S_{min} was set equal to zero. Moreover, to reduce the number of model parameters, k was fixed at 1/2 after testing and comparing a range of values. This reduces Eq. (5) to:

$$\mu(S(t)) = \mu_{opt} \cdot \frac{S(t) \cdot \sqrt{S(t) - S_{max}} \cdot \sqrt{S_{opt} - S_{max}}}{\left((S_{opt} - S_{max}) \cdot (S(t) - S_{opt}) - S_{opt} \cdot \left(S_{max} - {}^{1}/_{2} \cdot S_{opt} - {}^{1}/_{2} \cdot S(t)\right)\right)} \quad (6)$$

As such, the CPM has three parameters to be estimated, i.e., μ_{opt}, S_{opt} and S_{max}, meaning that it has the same level of model complexity as the Haldane equation.

Parameter estimation method

Model parameters were estimated using the function *lsqnonlin* of MATLAB R2021b. The optimal model parameters were determined that minimise the mean sum of squares of the residuals between the experimental measurements and corresponding model predictions. These residuals were weighted by dividing them by the width of the respective ranges of measurements of the corresponding datasets for glycerol, methanol and cells. As such, errors were weighted proportionally to the range of the data that was available to take into account that the different data types range over different scales.

3 Results and discussion

The first step in studying the kinetics of growth and substrate consumption of the wild type and genetically modified *P. pastoris* strains was to obtain experimental data on the evolution of viable cells and substrate in batch growth experiments. The experimental datasets are displayed in Fig. 1, Fig. 2, Fig. 4 and Fig. 5. These figures illustrate the high quality and quantity of the experimental data that was obtained. The data on glycerol and methanol concentrations demonstrate the wide range of concentrations that were covered during these experiments, providing information on the microbial kinetics. As such, this dataset is suitable

for constructing the desired mathematical models. The data has been used to construct mathematical models for both strains growing on both glycerol and methanol in Section 3.1. The microbial kinetics of the wild type and modified strain are then compared in Section 3.2 based on the obtained models.

3.1 Model development

The simple and generic mathematical model that is described in Eq. (1)-(2) was fitted to the datasets described in Section 2.1, based on the method described in Section 2.2. The parameter estimation was performed separately for glycerol and methanol as carbon sources and for the wild type and genetically modified *P. pastoris* strain. The performance of both the Monod and Haldane equation were compared to describe the effect of the substrate concentration on the microbial growth rate in each of the four datasets. The Monod equation assumes that growth rate always increases with an increasing substrate concentration whereas the Haldane equation describes an optimum substrate concentration that maximises the growth rate. For substrate concentrations above this optimum, the growth rate reduces asymptotically to zero.

For yeast growth on glycerol as the main carbon source, a comparison of the estimated model parameters with 95% confidence intervals is provided in Table 2, along with the Weighted MSE (WMSE) of the model fit. The resulting kinetic models are illustrated in Fig. 3a and b for the wild type and genetically modified strains, respectively. For the wild type strain, the model output of the Monod and Haldane equations are very similar. This is also seen from the parameter estimates where similar values are obtained for optimal growth rate. Moreover, the high value for the estimate of the inhibition constant (K_i) means that the quadratic term in the denominator of the equation is minimised, leading to the approximation of the Monod kinetics. When comparing the WMSE, it is seen that the Haldane equation achieved a slightly better approximation of the experimental data compared to the Monod

equation. Turning to the results of the genetically modified strain, Fig. 3b illustrates that the predicted growth rate while consuming glycerol differs more between the Monod and Haldane equation than it did for the wild type strain. The parameter estimation results in Table 2 demonstrate that the WMSE of the Haldane equation was about 20% lower than that of the Monod equation, signifying that the Haldane equation is more suitable to describe the observed kinetics. Knowing this, it becomes evident from Fig. 3b that the estimated Monod kinetics average out the Haldane kinetics in an attempt to achieve the best possible model fit with the given equation. For the genetically modified strain, it is clear that growth is inhibited at high glycerol concentrations, a phenomenon that cannot be described by the Monod equation. As such, when selecting a model to describe the effect of glycerol on the growth rate of *P. pastoris*, the Haldane equation is selected. Glycerol is known to be inhibitory to the growth of *P. pastoris* at high concentrations (Bushel et al., 2002). Since glycerol is added to the fermentation medium in large quantities as a main carbon source, its concentration has an effect on the water activity, i.e., the amount of free water that is available for microbial processes. Low water activity values, which occur at high substrate concentrations, are known to inhibit the growth rate of yeasts (Beuchat, 1983). However, because the amount of glycerol that is needed to significantly inhibit yeast growth is typically high, this phenomenon is not observed in all research. As such, other published models for the growth kinetics of *P. pastoris* on glycerol have often proposed the Monod equation (Barrigon et al., 2015., Canales et al., 2018; Ghosalkar et al., 2008). Using the Monod equation instead of the Haldane equation would have a significant effect on the outcome of a model-based optimization of a *P. pastoris* bioproduction process. Since the Monod equation assumes that the growth rate can only increase with increasing substrate concentration, assuming this model would justify adding the total quantity of glycerol required to produce the desired cell density at the beginning of the fermentation in a batch mode. The model-based optimization of the same process considering Haldane kinetics would lead to the

initiation of the process at the optimal concentration, followed by a fed-batch process. The latter is similar to the approach that is followed in standardised methods for recombinant protein production through *P. pastoris* (Invitrogen, San Diego, CA, U.S.A.). In these methods, yeast production is performed in a glycerol batch phase followed by a glycerol fed-batch phase to reduce the initial concentration of glycerol in the bioreactor, thereby increasing the average growth rate. The fit of the model using the Haldane equation to the experimental data is presented in Fig. 1 and Fig. 2, for the wild type and genetically modified strain, respectively. These figures indicate a good quality of fit of the selected model to the experimental data. The current results demonstrate that a kinetic model used to describe *P. pastoris* growth on glycerol as the main carbon substrate should include a form of inhibition at high glycerol concentrations, as can be described by the Haldane equation.

The parameter estimation results for *P. pastoris* growth on methanol as the main carbon source are provided in Table 3 for the Monod equation, Haldane equation and Cardinal Parameter Model (CPM). A first comparison between these parameter estimation results is done based on the WMSE. These results demonstrate that the Monod equation results in a high overall error on the model fit, meaning that it is unsuitable to describe the observed kinetics. The Monod equation assumes that the growth rate asymptotically increases to its optimal value for an increasing substrate concentration. However, methanol is known to be toxic at high concentrations for *P. pastoris* cells (Khatri and Hoffmann, 2006; Potvin et al., 2012). Even so, several authors have applied the Monod equation to describe *P. pastoris* growth on methanol (Canales et al., 2018; Curvers et al., 2002; Jahic et al., 2002). This simplification can only be valid in a very low range of methanol concentrations, where its toxicity is not yet observed. Given the current modelling results and fundamental knowledge on *P. pastoris'* behaviour, the Monod equation is clearly unsuitable for this application and will not be discussed further. The other two models that are considered are the Haldane equation and the CPM, which both have

an optimum concentration, above which the growth rate decreases. For both the wild type and genetically modified strain, the WMSE is lower for the CPM than for the Haldane equation. Therefore, the CPM, which is newly proposed for this application in the current study, is most suitable to describe the observed kinetics. The difference is most striking in case of the genetically modified strain. This is also observed in Fig. 6b, with a remarkable difference between the evolution of predicted growth rates according to both models. This is due to the very different structures of both models. In the Haldane equation, the growth rate reduces asymptotically to zero as the substrate concentration increases above its optimum. In practice, this means that the growth rate never becomes equal to zero. Contrary, in the CPM the growth rate reduces sharply to zero, which is reached at the maximum substrate concentration that supports growth. In case of the wild type strain, the Haldane equation is able to approximate the kinetics as predicted by the CPM up to about 25 mg/mL of methanol, but clearly fails to describe the toxicity that is observed at high methanol concentrations (Fig. 6a). The difference is even more pronounced in case of the genetically modified strain (Fig. 6b). Fig. 4 and Fig. 5 illustrate the fits of the dynamic model based on the CPM for the wild type and genetically modified strain, respectively. These figures demonstrate that a good fit to the experimental data was obtained. It can be concluded that the toxicity of methanol as incorporated in the CPM achieves a better description of the observed kinetics than the substrate inhibition as described in the Haldane equation.

During the parameter estimation, the yield of cells over substrate was estimated as a separate parameter for each experiment to observe any differences. The estimated yield parameters are summarised in Table 4 for both carbon sources and both strains as a function of the estimated initial substrate concentration. When comparing the results of both strains growing on glycerol and methanol, it is observed that the cell yield is much higher when *P. pastoris* grows on glycerol than on methanol. This finding also supports the common method

of first growing the cells on glycerol as a main carbon source and switching only to methanol when a sufficiently high cell density is reached. When comparing the results of both strains growing either on glycerol or on methanol, there are no specific differences noticed. This implies that the genetic modification did not affect the attained cell yield. When looking at the effect of the glycerol concentration on cell yield, it is remarkable to see that the yield is higher at the highest concentrations for both strains. When cells are produced at the optimal rate, a lower substrate concentration will be used, as discussed before. Consequently, the change of yield would not be noticed and an average of the observed yields at low and medium concentrations could be used to make predictions. However, if the objective is to achieve the highest possible cell density, it would be interesting to consider starting a batch process at a high substrate concentration instead of using a fed-batch mode. In the case of both strains growing on methanol as the main carbon source, no effects of the substrate concentration were found.

3.2 Wild type and GMO comparison

Both for growth on glycerol and methanol as the main carbon source, the kinetic model that was selected was the same for both the wild type and genetically modified strains. This indicates that the overall cell growth and substrate consumption behaviour of *P. pastoris* remained similar after the genetic modification. On the other hand, it was observed that all parameters of the selected kinetic models were statistically different both for growth on glycerol and methanol (Table 2 and Table 3). As such, while the overall trend of the kinetics remained the same, there was indeed a clear change observed in the macroscale behavioural parameters of *P. pastoris* due to the incorporation of the recombinant DNA. A more detailed comparison between the microbial kinetics of the wild type and genetically modified strain is obtained by interpreting Fig. 7.

For the yeast growth on glycerol (Fig. 7a), both the wild type and genetically modified strain have their optimal growth at low substrate concentrations. From the figure it can be observed that the specific growth rate of both strains reduced when glycerol concentration dropped to very low levels. This is also seen in both the data and simulations of Fig. 1 and Fig. 2, where growth suddenly stops when the substrate is completely consumed. The genetic modification has caused an increase in the optimal growth rate of about 17 %, which is most noticeable at very low concentrations of glycerol (Table 2). On the other hand, the inhibition due to high substrate concentrations has increased as well due to the genetic modification. A difference in growth pattern between the wild type and genetically modified strains is expected according to the report of Katakura et al. (1998) and Wong et al. (1998). One reason can be the change in the host cell physiology due to the addition of foreign DNA (Bentley,1990). In this study, the growth rate of the genetically modified strain was found to be higher than that of the wild type strain for glycerol concentrations up to about 40 mg/mL. Given that the glycerol batch process commonly starts at 40 mg/mL, this means that the growth rate of the genetically modified strain would be higher than that of a wild type strain within the typical relevant range. The fact that a pronounced optimum growth rate is observed for the genetically modified strain also means that there is a good opportunity for optimizing the glycerol batch and fed batch stages by finding an optimal glycerol feeding profile.

As seen from Fig. 7b in comparison with Fig. 7a, the genetic modification had an even more pronounced effect on the dynamics of *P. pastoris* when growing on methanol as carbon source. Also, for methanol, the optimal growth rate increased after the genetic modification. Moreover, in this case the optimum also shifted from low to moderate methanol concentrations. The most striking change after the genetic modification was the decrease in methanol tolerance. Specifically, the maximum methanol concentration that supports *P. pastoris* growth decreased from 34.5 mg/mL for the wild type to 27.7 mg/mL for the genetically modified strain (Table

3). It is this combination of an increase of the optimum substrate concentration with a decrease of the maximum substrate concentration that makes it impossible for the Haldane equation to provide a decent description of the microbial dynamics that were observed.

Looser et al. (2015) have reported that the maximum specific growth rates observed for engineered strains are generally lower than the non-engineered strains both for Mut^+ and Mut^s strains. Compared to *Saccharomyces cerevisiae*, most of the energy production from *P. pastoris* is through respiratory metabolism as it is a crab-tree negative organism. *P. pastoris* can grow 1.7 - 8.5 times faster on glycerol than on methanol (Looser et al., 2015). At high concentrations of glycerol, the genes responsible for methanol utilisation are repressed but are derepressed at substrate limiting conditions. On a cellular network level, the production of a foreign protein comes at an expense of energy due to consumption of resources that are required for normal cellular processes. This leads to changes in the central carbon metabolism resulting in reduced redox and energy cofactors thereby, creating inefficiencies in metabolism (Kafri et al., 2016; Klein et al., 2015; Wu et al., 2016). In a study conducted by Gopal et al. (1989), using *S. cerevisiae*, a reduction in biomass yield was observed for the genetically engineered strain in comparison to the control strain. The findings from the study indicated that the expression of a foreign protein created a competition for resources between host metabolism and gene expression leading to a reduction in biomass of the engineered strain. However, the biomass yield estimated in this study for both strains did not exhibit large differences for all the substrate concentrations investigated. In terms of growth rates, Krogh et al. (2008) identified a 33% reduction of maximal specific growth rate in genetically engineered *S. cerevisiae* compared to a wild type strain. These findings are in contrast to the observation of this study where an increase in specific growth rate has been achieved for the genetically modified strain when grown on both glycerol and methanol. Although *S. cerevisiae* has been used as a model organism to study yeast physiology, its metabolic functions can be different from that of *P.*

pastoris. Zhu et al. (2011) performed a transcriptional analysis to compare the metabolism of *P. pastoris* according to its gene dosage. The study indicated that the cellular physiology of a strain with high gene copies were affected. For instance, the specific growth rate declined prominently for strains having more than 12 gene copies (Zhu et al., 2009).

Li et al. (2019) elaborated in their work using transcriptomic analysis that the synthesis of amino acids was considerably reduced in genetically engineered *P. pastoris* when grown on methanol. Under the AOX1 promoter, methanol is used both as an inducer and carbon source for growth (Hartner et al., 2008). In the case of an engineered strain, during high protein expression, a metabolic shift is noticed towards increased generation of energy, reducing agents and networks such as the pentose phosphate pathway or TCA cycle, which causes the reduction of biomass yield (Nocon et al., 2016; Jordà et al., 2014). It has been reported that methanol is unable to compensate for both carbon and energy demands to achieve growth and recombinant protein production when supplied as a sole carbon source (Plantz et al., 2006). Hence, the shift in specific growth rate observed for genetically engineered strain as shown in Fig. 7b in comparison to the wild type strain might arise from the high demand for the substrate. Moreover, the cell physiology can be negatively affected due to overexpression of proteins, which directly leads to a low capacity for methanol consumption and reduced specific growth rate (Cos et al., 2005) or cell viability (Hohenblum et al., 2004). This enables the wild type strain to withstand higher concentrations of methanol in comparison to the genetically modified one. Certain proteins are required for methanol utilisation. These proteins are present in small amounts when the cells are grown on glycerol. However, when methanol is used as a sole carbon source, these proteins start to dominate the proteome (Tomàs-Gamisans et al., 2019). AOX alone constitutes about 30% of the total protein of the total soluble protein when methylotrophic yeast is grown on methanol. Additionally, the possibility for redistribution of carbon fluxes due to the production of a foreign protein takes place. The reduction in the growth

rate of both wild type and genetically modified *P. pastoris* at high concentrations can be explained by the toxicity imposed by methanol present in the medium. Specifically, methanol catabolism releases hydrogen peroxide (H_2O_2) and formaldehyde which are highly toxic through the oxidative stress they cause to the cells.

Factors responsible for improving the productivity of genetically engineered *P. pastoris* have been investigated by researchers over the years. Certain limitations in industrial bioprocesses such as long fermentation times, high substrate requirements and protein secretory factors degrading normal cell metabolism can greatly affect the production level of the proteins. Zhu et al. (2019) have reported on the possible bottlenecks associated with genetically engineered *P. pastoris* strains when used in industrial applications. These bottlenecks can arise due to factors such as the type of gene to be expressed and the promoters used. It has been identified that the metabolic flux distributions representing the cellular metabolic activities are influenced greatly by the type and amount of heterologous protein expressed in the host system. Also, the substrate utilisation and the range of specific growth rate and optimum productivity are dependent on the promoter used for protein expression. Hence, the overall optimisation of a protein production system is often difficult. Therefore, nowadays, researchers are focussing on customised process optimisation strategies for the specific strain used and the product required. The study and comparison of the growth and consumption behaviour of the wild type and genetically engineered strains in this study can lead to the optimisation of fed-batch *P. pastoris* bioprocesses.

4 Conclusions

This study investigates the growth pattern of *Pichia pastoris* when grown on glycerol or methanol in batch cultivations. Comparing the growth kinetics of a wild type and genetically modified strain on preferred substrates widens our understanding of the influence of genetic

manipulation on the metabolic behaviour of the host system. When grown on glycerol, *P. pastoris* exhibited the maximum growth rate at very low concentrations with a mild decrease in the growth rate towards higher concentrations as was described accurately by the Haldane model. Interestingly, the genetically modified strain showcased a higher growth rate than wild type strain, which has not been established yet in other published works. Moreover, the growth inhibition that was observed at high glycerol concentration cannot be described by Monod kinetics, even though it is a commonly used model for this purpose. In the case of growth on methanol, an optimal growth rate was clearly distinguished, above which the growth rate decreased sharply to zero. Since the commonly used Monod and Haldane kinetics cannot describe such behaviour, a Cardinal Parameter Model was successfully adopted to describe microbial growth on methanol for the first time. Based on its interpretable parameters, this model allowed to determine that the maximum methanol concentration that supports growth reduced due to the genetic modification, while the optimum concentration for growth increased. The observed shift in the optimum concentration can be explained by the higher demand for methanol by the genetically engineered strain for both growth and protein production. However, to establish a better understanding of the changes seen in the growth kinetics, estimation of intracellular fluxes is necessary. Overall, this study provides a better understanding of the cellular behaviour of the host organism on a macroscopic level and the mathematical model is the foundation for further process optimisation of recombinant protein production by *P. pastoris*. Furthermore, the growth kinetics determined for *P. pastoris* when grown on glycerol and methanol demonstrate the need to involve two carbon source phases during a fed-batch fermentation. *P. pastoris* growth in a methanol environment is (i) slower, (ii) more sensitive to changes in the substrate concentration and (iii) has a lower cell yield per quantity of substrate compared to glycerol.

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6 Author contributions

Conceptualisation, J.A.J., S.A. and J.F.M.V.I.; methodology, J.A.J., S.A. and J.F.M.V.I.; investigation, J.A.J. and S.A.; validation, J.A.J and S.A.; formal analysis, J.A.J. and S.A.; software, S.A.; resources, J.F.M.V.I.; data curation, J.A.J.; writing—original draft preparation, J.A.J and S.A.; writing—review and editing, J.A.J., S.A. and J.F.M.V.I.; visualisation, J.A.J., S.A. and J.F.M.V.I.; supervision, J.F.M.V.I.; funding acquisition, S.A. and J.F.M.V.I. All authors have read and agreed to the published version of the manuscript.

7 Conflict of interest

The authors have no conflict of interest to declare.

8 Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figures

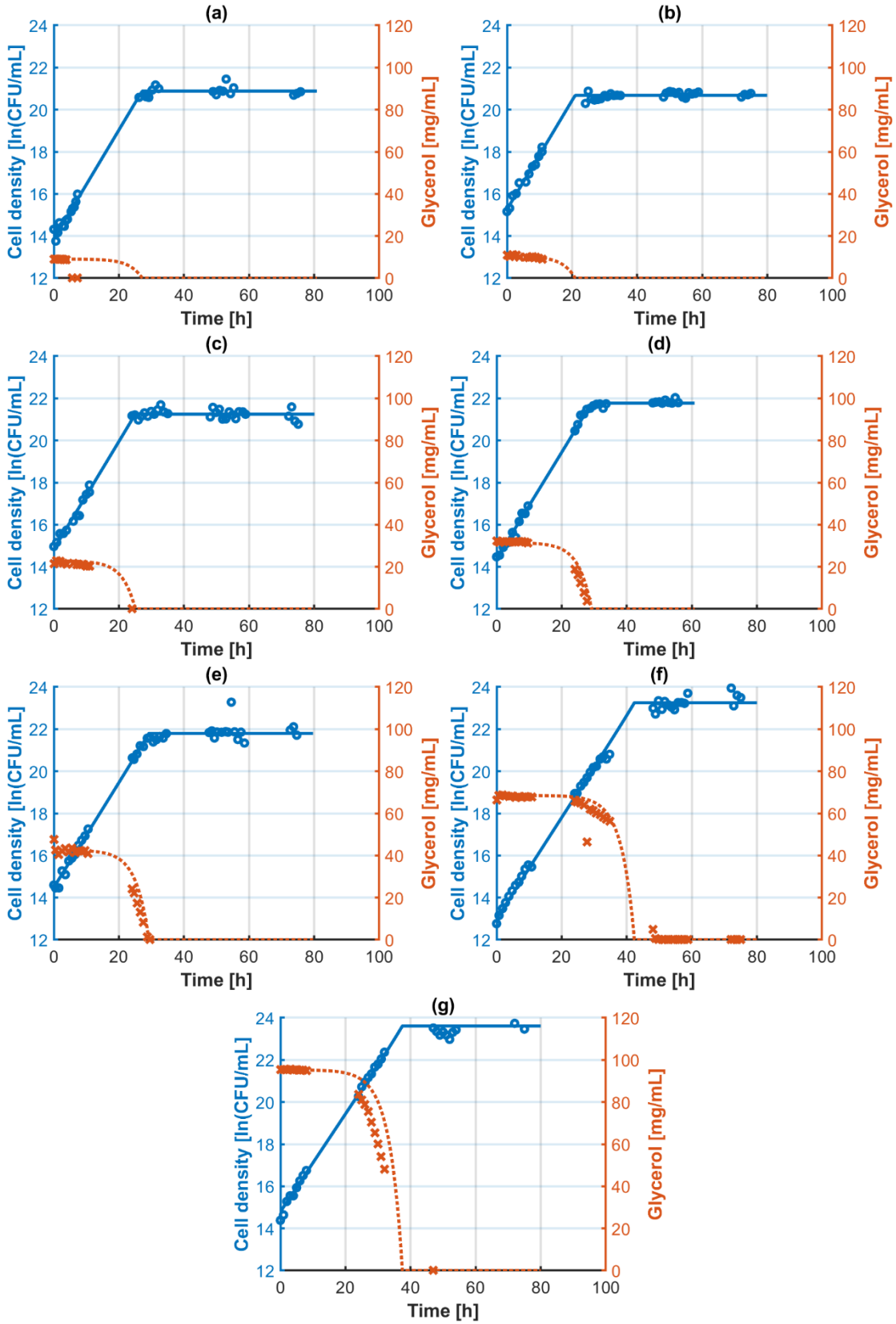

Fig. 1: Measured dynamics of cell growth (o) and glycerol consumption (x) of the wild type *P. pastoris* and model approximation using the Haldane equation (— and ---).

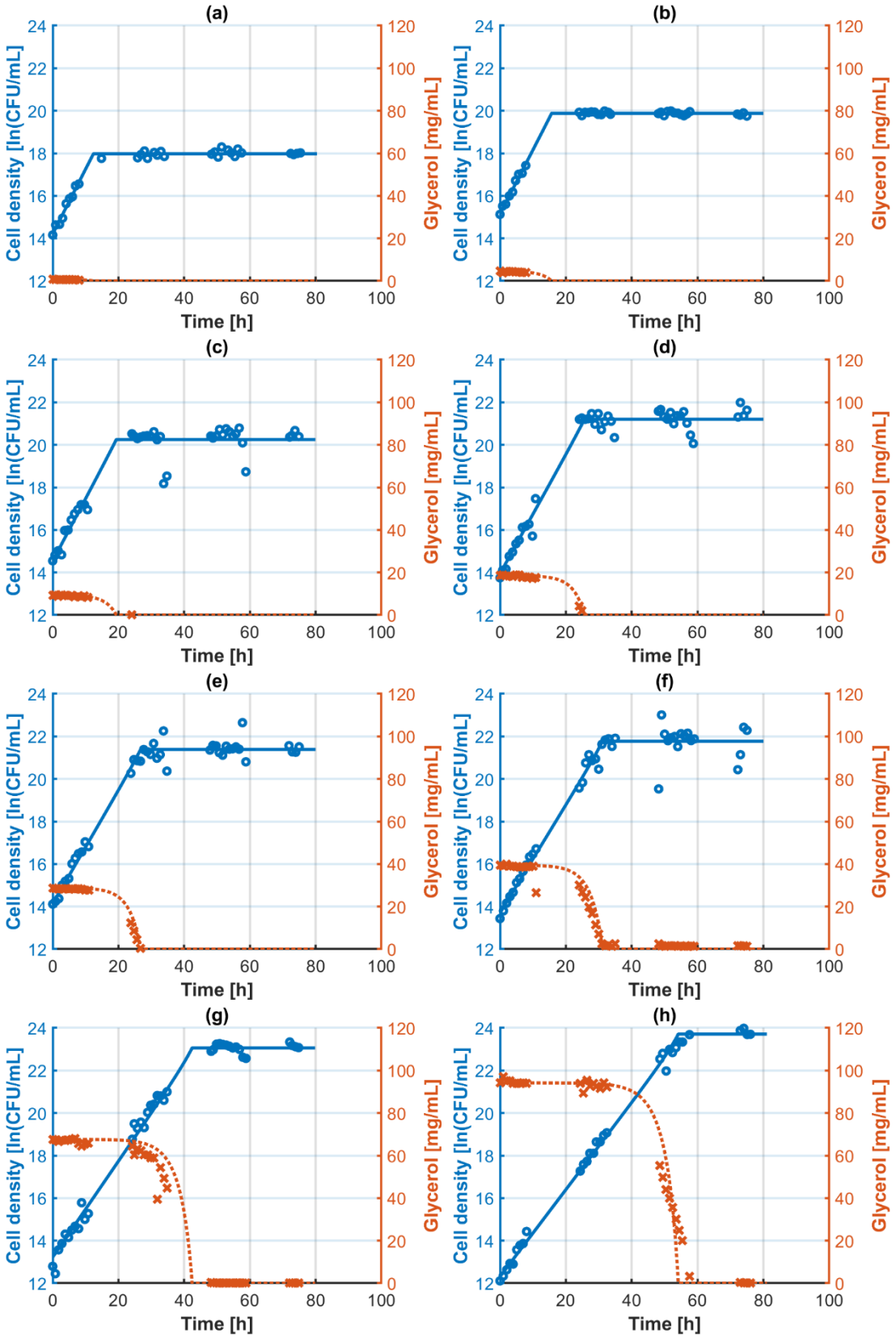

Fig. 2: Measured dynamics of cell growth (o) and glycerol consumption (x) of the genetically modified *P. pastoris* and model approximation using the Haldane equation (— and ---).

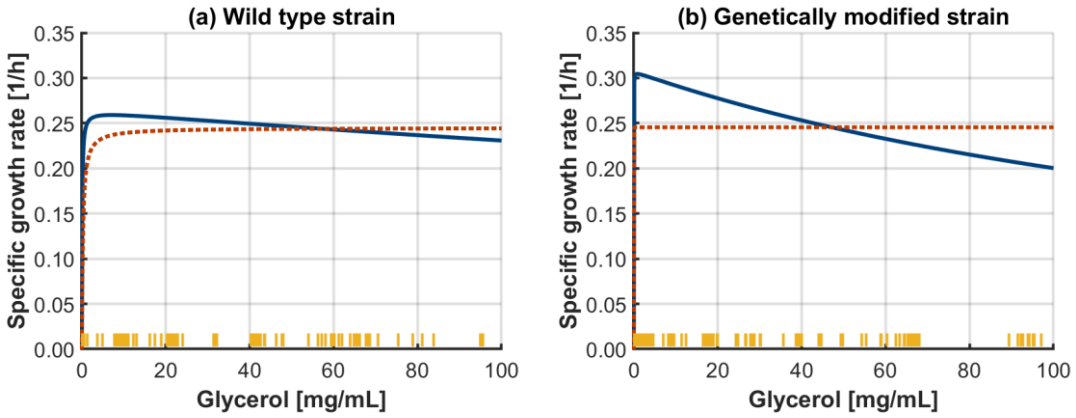

Fig. 3: Kinetic models that are obtained from fitting the model in Eq. (3)-(4) to the experimental data on the growth of the (a) wild type and (b) genetically modified strain on glycerol. The kinetic models that are illustrated are the Monod equation (---) and Haldane equation (—). Vertical bars (|) represent all measured concentrations of the substrate during the growth experiments.

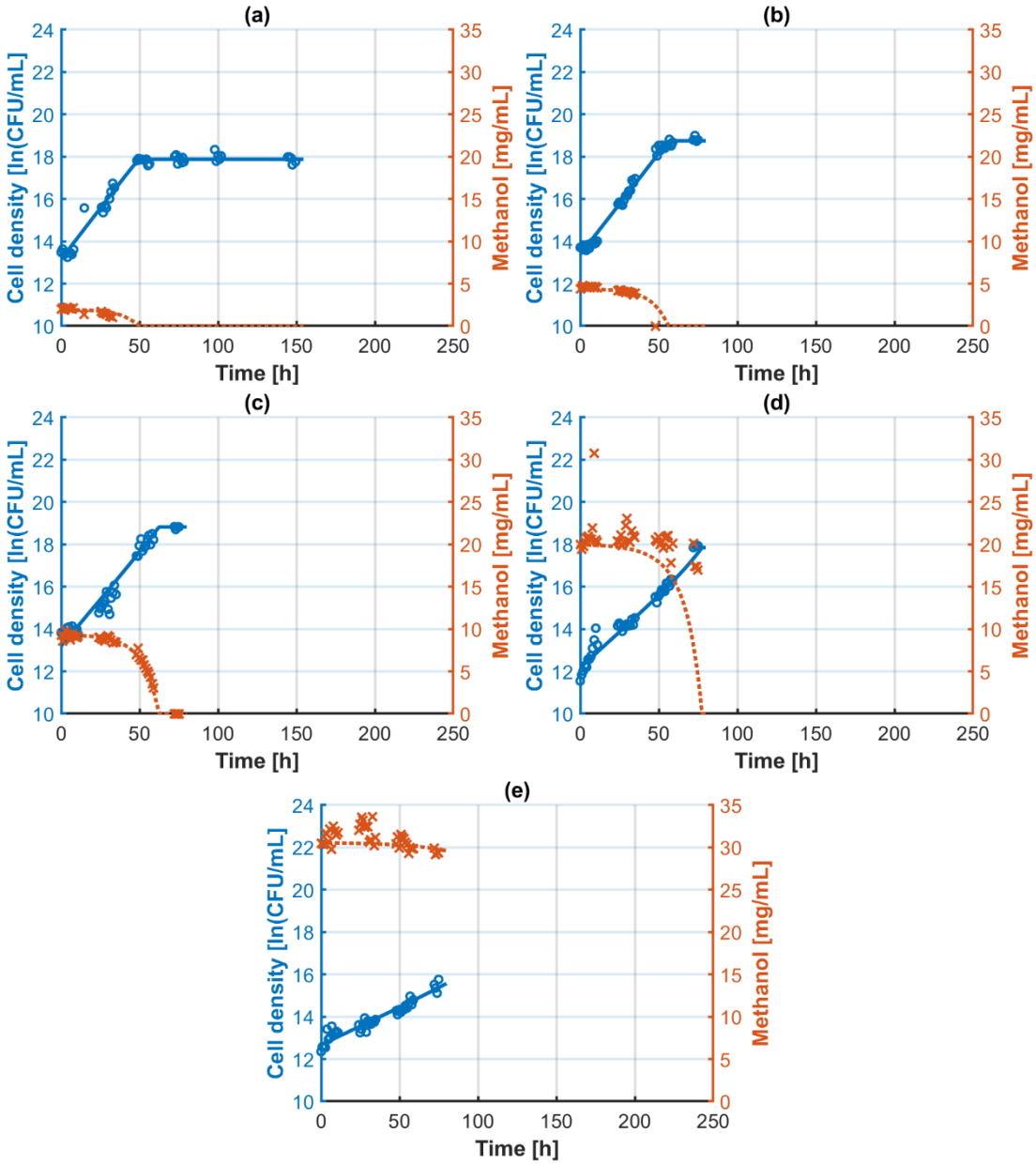

Fig. 4: Measured dynamics of cell growth (**o**) and methanol consumption (**x**) of the wild type *P. pastoris* and model approximation using the CPM (**—** and **---**).

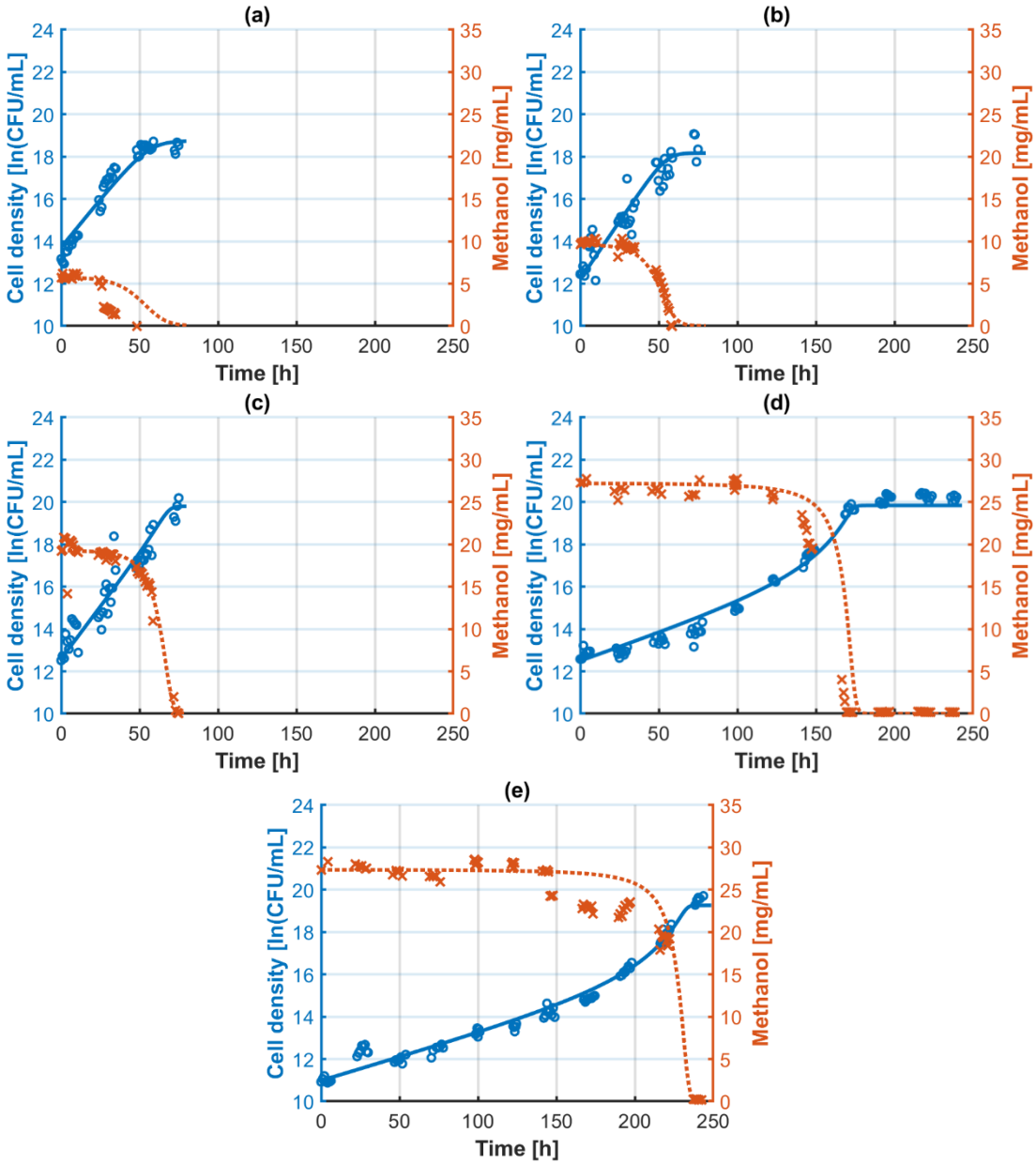

Fig. 5: Measured dynamics of cell growth (o) and methanol consumption (x) of the genetically modified *P. pastoris* and model approximation using the CPM (— and ---).

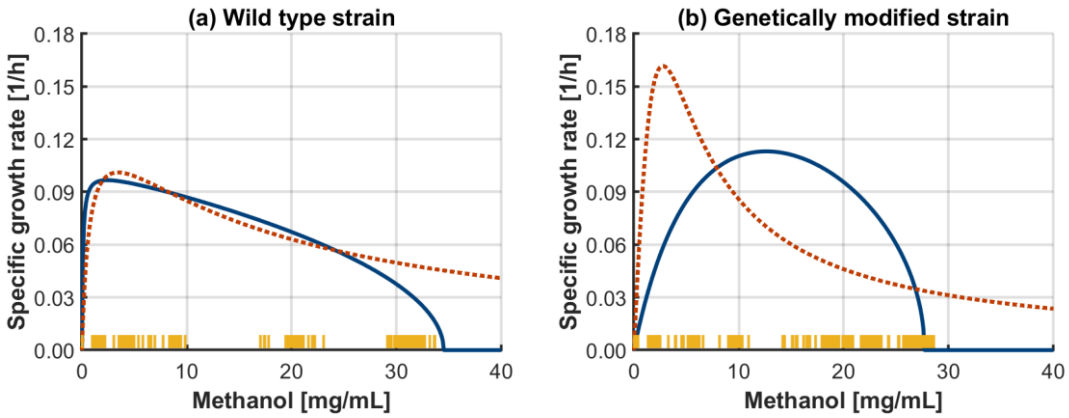

Fig. 6: Kinetic models that are obtained from fitting the model in Eq. (4)-(6) to the experimental data on the growth of the (a) wild type and (b) genetically modified strain on Methanol. The kinetic models that are illustrated are the Haldane equation (---) and cardinal parameter model (—). Vertical bars (|) represent all measured concentrations of the substrate during the growth experiments.

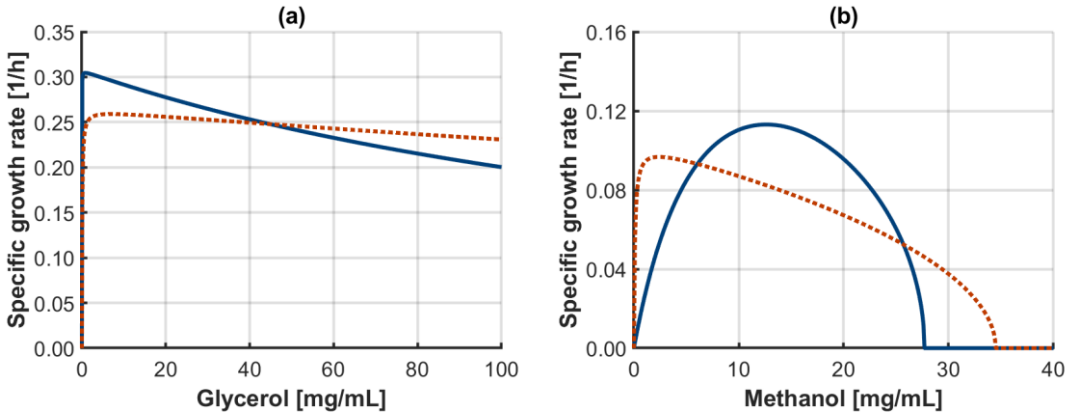

Fig. 7: Comparison of the kinetic models that are obtained from fitting the model in Eq. (3)-(6) to the experimental data on the growth on (a) glycerol and (b) methanol for both the wild type (---) and genetically modified strain (—). The Haldane equation is used to describe growth on glycerol and the CPM for growth on methanol.

Tables

Table 2: Parameter estimates and 95 % confidence bounds of the kinetic models for the growth of both the wild type and genetically modified strain on glycerol as main carbon source. Superscript lower case letters signify the existence or absence of significant differences between the estimated parameters for the wild type and genetically modified strain according to a one-way ANOVA. The Weighted MSE (WMSE) indicates the quality of the model fit.

	Wild type	**Genetically modified**
Parameters	**Monod equation**	
μ_{opt} [1/h]	0.245[a] [0.243; 0.247]	0.245[a] [0.244; 0.247]
K_s [mg/mL]	0.24[a] [0.04; 0.44]	0.00[b] [-0.04; 0.04]
WMSE	$2.97 \cdot 10^{-4}$	$9.08 \cdot 10^{-4}$
Parameters	**Haldane equation**	
μ_{opt} [1/h]	0.259[a] [0.253; 0.265]	0.304[b] [0.293; 0.316]
K_m [mg/mL]	0.07[a] [-0.26; 0.40]	0.00[b] [-0.03; 0.04]
K_i [mg/mL]	693.12 [a] [500.12; 886.11]	187.28 [b] [178.31; 196.24]
WMSE	$2.80 \cdot 10^{-4}$	$7.29 \cdot 10^{-4}$

Table 3: Parameter estimates and 95 % confidence bounds of the kinetic models for the growth of both the wild type and genetically modified strain on methanol as main carbon source. Superscript lower case letters signify the existence or absence of significant differences between the estimated parameters for the wild type and genetically modified strain according to a one-way ANOVA. The Weighted MSE (WMSE) indicates the quality of the model fit.

	Wild type	**Genetically modified**
Parameters	**Monod equation**	
μ_{opt} [1/h]	0.069[a] [0.068; 0.070]	0.038[b] [0.038; 0.038]
K_s [mg/mL]	0.00[a] [-0.05; 0.05]	0.00[a] [-0.05; 0.05]
WMSE	$3.34 \cdot 10^{-3}$	$1.29 \cdot 10^{-2}$
Parameters	**Haldane equation**	
μ_{opt} [1/h]	0.101[a] [0.100; 0.102]	0.162[b] [0.151; 0.172]
K_m [mg/mL]	0.75[a] [0.59; 0.91]	20.84[b] [-19.04; 60.73]
K_i [mg/mL]	15.82[a] [14.29; 17.35]	0.37[b] [-0.23; 0.97]
WMSE	$1.31 \cdot 10^{-3}$	$4.70 \cdot 10^{-3}$
Parameters	**Cardinal parameter model**	
μ_{opt} [1/h]	0.097[a] [0.095; 0.098]	0.113[b] [0.112; 0.114]
S_{opt} [mg/mL]	2.40[a] [1.70; 3.10]	12.62[b] [12.49; 12.76]
S_{max} [mg/mL]	34.49[a] [34.07; 34.91]	27.70[b] [27.64; 27.76]
WMSE	$9.41 \cdot 10^{-4}$	$1.67 \cdot 10^{-3}$

Table 4: Parameter estimates and 95 % confidence bounds of the yield parameters of each experiment as a function of the estimated initial substrate concentration. Superscript lower case letters signify the existence or absence of significant differences between the estimated yield parameters within a combination of carbon source and yeast strain according to a one-way ANOVA.

Glycerol				**Methanol**			
Wild type		**Genetically modified**		**Wild type**		**Genetically modified**	
$S_{G,0}$ [mg/mL]	$Y_{X/S}$ [10^8 CFU/mg]	$S_{G,0}$ [mg/mL]	$Y_{X/S}$ [10^8 CFU/mg]	$S_{M,0}$ [mg/mL]	$Y_{X/S}$ [10^7 CFU/mg]	$S_{M,0}$ [mg/mL]	$Y_{X/S}$ [10^7 CFU/mg]
9.05	1.30^{a} [1.22; 1.36]	0.76	0.83^{a} [0.79; 0.87]	2.03	0.74^{a} [0.16; 0.34]	5.75	2.39^{a} [2.25; 2.54]
10.26	0.93^{b} [0.90; 0.97]	4.54	0.94^{b} [0.90; 0.98]	4.45	2.90^{b} [2.81; 3.04]	9.58	0.81^{b} [0.78; 0.85]
22.78	0.74^{c} [0.71; 0.77]	9.29	0.67^{c} [0.64; 0.70]	9.29	3.04^{b} [2.89; 3.23]	19.25	2.08^{c} [1.98; 2.16]
31.42	0.91^{d} [0.87; 0.95]	18.47	0.88^{d} [0.84; 0.90]	19.96	1.62^{c} [1.54; 1.69]	27.20	1.52^{d} [1.47; 1.57]
42.52	0.68^{e} [0.66; 0.71]	28.77	0.67^{c} [0.65; 0.70]	30.56	0.19^{d} [0.18; 0.20]	27.35	0.85^{e} [0.82; 0.88]
68.51	1.84^{f} [1.75; 1.91]	39.47	0.73^{e} [0.70; 0.75]				
95.26	1.88^{g} [1.80; 1.97]	67.62	1.54^{f} [1.46; 1.60]				
		94.17	2.12^{g} [2.01; 2.22]				