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P_{AOX1} expression in mixed-substrate continuous cultures of *Komagataella phaffii* (*Pichia pastoris*) is completely determined by methanol consumption regardless of the secondary carbon source

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The expression of recombinant proteins by the *AOX1* promoter of *Komagataella phaffii* is typically induced by adding methanol to the cultivation medium. Since growth on methanol imposes a high oxygen demand, the medium is often supplemented with an additional secondary carbon source which serves to reduce the consumption of methanol, and hence, oxygen. Early research recommended the use of glycerol as the secondary carbon source, but more recent studies recommend the use of sorbitol because glycerol represses P_{AOX1} expression. To assess the validity of this recommendation, we measured the steady state concentrations of biomass, residual methanol, and LacZ expressed from P_{AOX1} over a wide range of dilution rates (0.02–0.20 h^{-1}) in continuous cultures of the Mut^+ strain fed with methanol + glycerol (repressing) and methanol + sorbitol (non-repressing). We find that under these conditions, the specific P_{AOX1} expression rate (measured as either specific LacZ productivity or specific AOX productivity) is completely determined by the specific methanol consumption rate regardless of the type (repressing/non-repressing) of the secondary carbon source. In both cultures, the specific P_{AOX1} expression rate is proportional to the specific methanol consumption rate, provided that the latter is below 0.15 g/(gdw-h); beyond this threshold consumption rate, the specific P_{AOX1} expression rate of both cultures saturates to the same value. Analysis of the data in the literature shows that the same phenomenon also occurs in continuous cultures of *Escherichia coli* fed with mixtures of lactose plus repressing/non-repressing carbon sources. The specific P_{lac} expression rate is completely determined by the specific lactose consumption rate, regardless of the type of secondary carbon source, glycerol or glucose.

KEYWORDS

***Komagataella phaffii* (*Pichia pastoris*), recombinant protein, glycerol, sorbitol, methanol, methanol consumption rate**

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Introduction

The methylotrophic yeast *Komagataella phaffii*, referred to earlier as *Pichia pastoris* (Kurtzman, 2005; Kurtzman, 2009), is a popular expression host (Schwarzhans et al., 2016; Rahimi et al., 2019a; Ergün et al., 2021). There are several reasons for this, but the most important one is that *K. phaffii* has an unusually strong and tightly regulated promoter which drives the expression of alcohol oxidase (AOX) in the presence of methanol (Higgins and Cregg, 1998; Ahmad et al., 2014; Gasser and Mattanovich, 2018). To be sure, *K. phaffii* has two alcohol oxidase genes, *AOX1* and *AOX2*, with corresponding promoters, P_{AOX1} and P_{AOX2}, but P_{AOX1} is used to drive recombinant protein expression since it is ~10 times stronger than P_{AOX2} (Cregg et al., 1989).

In the first expression system constructed with *K. phaffii*, the wild-type strain was used as host, and recombinant protein was expressed under the control of P_{AOX1} by using methanol as inducer (Cregg et al., 1985). Although this Mut$^+$ (methanol utilization plus) strain yielded excellent recombinant protein expression, the use of methanol as inducer led to several operational problems (Macauley-Patrick et al., 2005; Cos et al., 2006; Jahic et al., 2006; Jungo et al., 2007a; Arnau et al., 2011; Potvin et al., 2012; Yang and Zhang, 2018; García-Ortega et al., 2019; Liu et al., 2019). Indeed, methanol is inflammable which poses safety issues (Liu et al., 2022). Moreover, methanol metabolism results in high oxygen demand and heat generation, as well as excretion of toxic metabolites, such as formaldehyde, that inhibit growth (Jungo et al., 2007b; Juturu and Wu, 2018; Rahimi et al., 2019b).

The problems stemming from the use of methanol as inducer led to several strategies for reducing methanol consumption (Feng et al., 2022). One strategy was to engineer the host strain by deleting either *AOX1* or both *AOX1* and *AOX2*, thus producing the Mut^s (methanol utilization slow) and Mut$^-$ (methanol utilization minus) strains, respectively, whose capacity to consume methanol is substantially impaired or abolished (Chiruvolu et al., 1997). Another strategy was to introduce into the medium, in addition to the *primary* or *inducing* carbon source methanol, a *secondary* or *non-inducing* carbon source that supports growth but not induction (Ergün et al., 2021). This reduces methanol consumption due to the sparing effect of the secondary carbon source, and increases the volumetric productivity due to the enhanced cell growth derived from metabolism of the secondary carbon source (Brierley et al., 1990; Egli and Mason, 1991; Jungo et al., 2007a; Jungo et al., 2007b; Paulova et al., 2012).

The foregoing strategies have led to reduced methanol consumption, but they can also result in decreased recombinant protein expression. Recently, we found that host strain engineering decreases recombinant protein expression substantially—the specific productivities of the engineered Mut^s and Mut$^-$ strains are respectively 5- and 10-fold lower than that of the Mut$^+$ strain (Singh and Narang, 2020). Since these three strains differ only with respect to their capacity for methanol consumption, the methanol consumption rate is an important determinant of the P_{AOX1} expression rate.

The goal of this work is to quantify the extent to which P_{AOX1} expression is affected by addition of a secondary carbon source to the medium. It is commonly held that this is determined by the type of the secondary carbon source. Specifically, these carbon sources have been classified as *repressing* or *non-repressing* based on the P_{AOX1} expression levels observed in *batch* cultures of the Mut$^-$ strain grown on mixtures of methanol and various secondary carbon sources (Inan and Meagher, 2001). Repressing carbon sources, such as glycerol, abolish P_{AOX1} expression, whereas non-repressing carbon sources, such as sorbitol, permit P_{AOX1} expression. The same conclusion has been reached from studies of mixed-substrate growth in fed-batch cultures (Brierley et al., 1990; Thorpe et al., 1999; Xie et al., 2005; Çelik et al., 2009; Wang et al., 2010; Gao et al., 2012; Niu et al., 2013; Carly et al., 2016; Azadi et al., 2017; Chen et al., 2017) and continuous cultures (Jungo et al., 2006; Jungo et al., 2007a; Jungo et al., 2007b; Canales et al., 2015; Berrios et al., 2017). Indeed, even though glycerol is commonly used as the secondary carbon source, the use of sorbitol has been almost unanimously recommended on the grounds that glycerol represses P_{AOX1} expression.

Most of the comparative studies cited above used constant fed-batch cultures, but these data can be difficult to interpret physiologically, because the specific growth rate decreases throughout the course of the experiment (Nieto-Taype et al., 2020). The comparative studies with continuous cultures are reviewed at length in the Discussion. Here, it suffices to note that many of these studies were performed at a fixed dilution rate D, and hence, specific growth rate (Jungo et al., 2007a; Jungo et al., 2007b; Berrios et al., 2017). We reasoned that comparative studies over a wide range of D could yield deeper physiological insights into the factors governing P_{AOX1} expression. Moreover, the optimal operating conditions determined in continuous cultures can also inform optimal protein production in exponential fed-batch cultures (Jungo et al., 2007a; Jungo et al., 2007b).

We were therefore led to study P_{AOX1} expression in continuous cultures of *K. phaffii* operated at various dilution rates with fixed concentrations of methanol + glycerol and methanol + sorbitol. To this end, we used a Mut$^+$ strain expressing LacZ from P_{AOX1}, but we also measured the AOX level to check the consistency of the data. We find that the specific P_{AOX1} expression rate (measured as either specific LacZ productivity or specific AOX productivity) is completely determined by the specific methanol consumption rate, regardless of the type (repressing/non-repressing) of the secondary carbon source.

Materials and methods

Microorganism and growth medium

A *K. phaffii* Mut$^+$ strain, GS115 (*his4*), was procured from J. M. Cregg, Keck Graduate Institute, Claremont, CA, United States and was genetically modified to express a recombinant β-galactosidase protein. Details of the strain construction have been presented elsewhere (Singh and Narang, 2020). The resulting strain was called Mut$^+$ (pSAOH5-T1) and was used for this study. Stock cultures were stored in 25% glycerol at −80°C.

The minimal medium composition used for shake-flask as well as chemostat cultivations was chosen such as to ensure stoichiometric limitation of the carbon and energy sources, as described in Egli and Fiechter (1981). The defined medium was supplemented with either glycerol (~3.1 g L^{-1}), a mixture of methanol (~1.6 g L^{-1}) and glycerol (~1.5 g L^{-1}) or a mixture of methanol (~3.2 g L^{-1}) and sorbitol (~1.5 g L^{-1}) as carbon sources. In addition, the medium contained

100 mM phosphate buffer (pH 5.5), 15.26 g NH_4Cl, 1.18 g $MgSO_4·7H_2O$, 110 mg $CaCl_2·2H_2O$, 45.61 mg $FeCl_3$, 28 mg $MnSO_4·H_2O$, 44 mg $ZnSO_4·7H_2O$, 8 mg $CuSO_4·5H_2O$, 8.57 mg $CoCl_2·6H_2O$, 6 mg $Na_2MoO_4·2H_2O$, 8 mg H_3BO_3, 1.2 mg KI, 370 mg EDTA disodium salt, 2.4 mg biotin per liter. All components of the defined medium were prepared and sterilised by either filtration or autoclaving as separate stock solutions and then mixed before cultivation.

Inoculum preparation and chemostat cultivation

When required, cells were revived in a 100 ml shake flask containing 10 ml minimal medium supplemented with a suitable carbon source at 30°C and 200 rpm. These primary cultures were sub-cultured once before inoculating the reactor precultures (in the same cultivation medium as prepared for the reactor vessel), which were then used as an inoculum for the bioreactor.

Chemostat cultivations were performed using bench-scale 0.5 L mini bioreactors modified to support chemostat operation and equipped with pH, DO, temperature, level and agitation controls (Applikon Biotechnology, Netherlands) at working volumes of 0.3 L. The cultivation temperature was always maintained at 30°C and pH at 5.5 by the automatic addition of 2 M NaOH. An integrated mass flow controller ensured a constant supply of air to the reactor vessel at 80 ml min^{-1}. Dissolved oxygen levels were monitored by a polarographic probe calibrated with respect to an air-saturated medium. Cultures were agitated to ensure fast mixing as well as aerobic conditions, such that the DO level always remained above 60%. A silicone based anti-foam agent was added to the reactor vessel as and when required to prevent foam formation and wall growth. For chemostat mode operation, the dilution rate was set by fixing the input feed flow rate, while a constant volume was maintained inside the reactor vessel by controlling the output feed flow rate *via* proportional control based on the on-line monitoring of the change in weight of the reactor vessel. For instance, for a dilution rate of 0.1 h^{-1}, the input feed flow rate was fixed at 30 ml h^{-1} using a peristaltic pump. When the weight of the reactor vessel increased beyond the set point, the output feed pump was switched on to remove the excess volume. After inoculation, cells were grown in batch phase for some time to allow exhaustion of the initial carbon source (indicated by a rise in DO level), followed by initiating the input and output feed supplies. At any particular dilution rate, steady-state samples were withdrawn after 5-6 liquid residence times. In general, three samples were collected for each dilution rate, separated by an interval of one liquid residence time. For instance, at a dilution rate of 0.04 h^{-1}, the first sample was taken after 150 h (6 liquid residence time), the second after 175 h (7 liquid residence time) and the third after 200 h (8 liquid residence time). Attainment of steady-state was confirmed by analysing the samples for constant dry cell weight and specific enzyme activities.

Sample collection and processing

For determination of residual substrate concentration inside the reactor, samples were withdrawn directly from the vessel. To achieve rapid biomass separation, culture samples were withdrawn using vacuum through a sampling tube attached to a 0.2-micron syringe filter and stored at −20°C until analysis. Samples for determination of biomass and enzyme activities were collected in a sampling bottle kept on ice. Biomass samples were processed immediately, while samples for measuring enzyme activities were pelleted, washed and stored at −20°C until processing.

Substrate analysis

Glycerol and sorbitol concentrations were estimated by high-performance liquid chromatography (HPLC) analysis (1100 series, Agilent Technologies, Palo Alto, United States) with detection limits of ~1 mg/L and ~30 mg/L. An ion-exclusion chromatography column from Phenomenex, California, United States (ROA-Organic acid H^+ column, 300 × 7.8 mm, 8 μm particle size, 8% cross linkage) with a guard column (Carbo-H cartridges) was used with 5 mM H_2SO_4 in ultrapure water as mobile phase supplied at a constant flow rate of 0.5 ml min^{-1}. The column chamber was maintained at 60°C and a refractive index detector was used for substrate measurement. Methanol concentrations were determined with a gas chromatograph equipped with a flame ionisation detector (GC-FID) (7890A, Agilent Technologies, Palo Alto, United States) using a HP-PLOT/Q column (30 m × 0.32 mm, 20 μm) from Agilent Technologies and nitrogen as the carrier gas. The detection limit for methanol was ~5 mg/L.

Dry cell weight measurement

A known volume of the fermentation broth was collected and pelleted in a pre-weighed centrifuge tube. Pellets were washed twice with distilled water and then dried at 80°C to constant weight.

Cell-free extract preparation

Culture samples were collected on ice and immediately centrifuged at 4°C to collect cells. The cell pellets were washed twice with phosphate buffer (100 mM, pH 7.4) and stored at −20°C until analysis. For cell lysis, pellets were resuspended in 100 μL of chilled breaking buffer (Jungo et al., 2006). Acid-washed glass beads (0.40–0.45 mm diameter) were added to the resulting slurry followed by alternate vortexing (1 min) and resting (on ice for 1 min) steps. This cycle was repeated 4–5 times, after which the cell debris was removed by centrifugation. Cell-free extracts (supernatant) were collected in fresh tubes kept on ice and immediately used for the estimation of enzyme activities. The Bradford assay was used for the estimation of the total protein content of the cell-free extracts for which bovine serum albumin served as standard (Bradford, 1976).

β-galactosidase assay

β-galactosidase assays were performed according to the method described by Miller (1972) with modifications. Briefly, cell-free extracts were appropriately diluted and mixed with Z-buffer

containing β-mercaptoethanol (Miller, 1972) and incubated at 30°C in a water-bath for 15–20 min. The reaction was started by adding ONPG and stopped by adding Na_2CO_3 when sufficient colour had developed. The specific β-galactosidase activity was calculated with the formula

$$1000 \times \frac{\mathrm{OD}_{420}/\text{Reaction time (min)}}{\text{Protein concentration in extract}\left(\frac{\mathrm{mg}}{\mathrm{ml}}\right) \times \text{Sample volume (ml)}}$$

and expressed in units mgp^{-1} where mgp denotes mg of total protein.

Alcohol oxidase assay

Appropriate dilutions of the cell-free extracts were used to measure alcohol oxidase activities based on the method adapted from Jungo et al. (2006). A fresh 2x stock of the assay reaction mixture containing 0.8 mM 4-aminoantipyrine, 50 mM phenolsulfonic acid, freshly prepared 4 U/ml horseradish peroxidase in potassium phosphate buffer (200 mM, pH 7.4) was prepared before setting up the assays. 100 μl of the diluted cell-free extracts were mixed with 25 μl methanol and incubated at 30°C for 10 min. After this, 100 μl of the 2x reaction mixture stock was added to the mix at time t = 0 to start the reaction and the increase in absorbance at 500 nm was monitored every 30 s for 10 min using a microplate reader (SpectraMax M2e, Molecular Devices Corporation, CA, United States). The specific alcohol oxidase activity was calculated with the formula

$$100,000 \times \frac{\mathrm{OD}_{500}/\text{Reaction time (s)}}{\text{Protein concentration in extract}\left(\frac{\mathrm{mg}}{\mathrm{ml}}\right) \times \text{Sample volume (ml)}}$$

and reported in units mgp^{-1}.

Calculating substrate consumption and protein productivities from the data

We are concerned with experiments in which a chemostat is fed with the primary carbon source S_1 (methanol) and a secondary carbon source S_2 which may be repressing (glycerol) or non-repressing (sorbitol). The primary carbon source S_1 induces the synthesis of the enzyme E_1 which represents LacZ or AOX, since the latter is expressed almost entirely from an *AOX1* promoter. We are interested in measuring the steady state concentrations of biomass X, primary carbon source S_1, and secondary carbon source S_2, as well as the specific activity of enzyme E_1. These quantities are denoted x, s_1, s_2, and e_1, respectively, and satisfy the mass balances:

$$0 = \frac{dx}{dt} = -Dx + \mu x \quad (1)$$

$$0 = \frac{ds_1}{dt} = D\left(s_{f,1} - s_1\right) - r_{s,1}x \quad (2)$$

$$0 = \frac{ds_2}{dt} = D\left(s_{f,2} - s_2\right) - r_{s,2}x \quad (3)$$

$$0 = \frac{de_1}{dt} = r_{e,1} - \mu e_1 \quad (4)$$

where $s_{f,1}$, $s_{f,2}$ denote the respective feed concentrations of S_1, S_2; and μ, $r_{s,1}$, $r_{s,2}$, $r_{e,1}$ denote the respective specific rates of growth, consumption of substrate, and expression of a stable intracellular protein (Pfeffer et al., 2011; Singh and Narang, 2020). It follows from Eqs 1–4 that

$$r_{s,i} = \frac{D\left(s_{f,i} - s_i\right)}{x}, i = 1, 2 \quad (5)$$

$$r_{e,1} = De_1 \quad (6)$$

These equations were used to calculate $r_{s,1}$, $r_{s,2}$, and $r_{e,1}$ from the measured values of the operating conditions D, $s_{f,i}$ and the steady state concentrations s_i, x, and e_1.

Results

Substrate consumption and P_{AOX1} expression in the presence of glycerol and sorbitol

Our goal is to study the kinetics of substrate consumption and P_{AOX1} expression during mixed-substrate growth on methanol + glycerol and methanol + sorbitol; however, we also characterized the substrate consumption kinetics during single-substrate growth on glycerol and sorbitol. In batch (shake-flask) cultures grown on glycerol and sorbitol, the biomass yields were quite similar (~0.6 gdw g^{-1}), but the maximum specific growth rates μ_m were dramatically different (Table 1). Due to the exceptionally small μ_m of 0.03 h^{-1} on sorbitol, we could not perform chemostat experiments with pure sorbitol, but we did perform such experiments with glycerol. We found that the biomass and residual glycerol concentrations followed the pattern characteristic of single-substrate growth in continuous cultures (Figure 1A). The specific glycerol consumption rate, calculated from these data using Eq. 5, increased linearly with D with a significant positive y-intercept (Figure 1B). Fitting these data to Pirt's model (Pirt, 1965) gave a true biomass yield of 0.67 gdw g^{-1}, and specific maintenance rate of 0.07 g gdw^{-1} h^{-1}. The specific LacZ and AOX activities, which were positively correlated in general, are inversely proportional to D, except for the two data points at the largest D (Figure 1C). This implies that the specific productivity is constant at all but the two largest D (Figure 1D), and the sharp decline at the two largest D may reflect the onset of regulation. Nevertheless, the specific LacZ and AOX productivities, calculated from the data in Figure 1C using Eq. 6, did not exceed ~1000 and ~300 units mgp^{-1} h^{-1}, respectively (Figure 1D).

Substrate consumption and P_{AOX1} expression in the presence of mixtures

When the Mut^{+} strain is grown in batch cultures of methanol + glycerol and methanol + sorbitol, there is diauxic growth, but methanol is the *unpreferred* substrate during growth on methanol + glycerol, and the *preferred* substrate during growth on methanol + sorbitol (Ramón et al., 2007). Such mixtures, which display diauxic growth in batch cultures, exhibit a characteristic substrate

TABLE 1 Maximum specific growth rates and biomass yields during single-substrate growth of the Mut⁺ strain of *K. phaffii* on glycerol and sorbitol. The true biomass yield in the chemostat was determined by fitting the variation of the specific substrate consumption rate with D to Pirt's model.

Carbon source	Maximum specific growth rate (h^{-1})	Biomass yield in shake flask (gdw g^{-1})	True biomass yield in chemostat (gdw g^{-1})
Glycerol	0.24 ± 0.01	0.61 ± 0.03	0.67
Sorbitol	0.03 ± 0.01	0.56 ± 0.01	ND

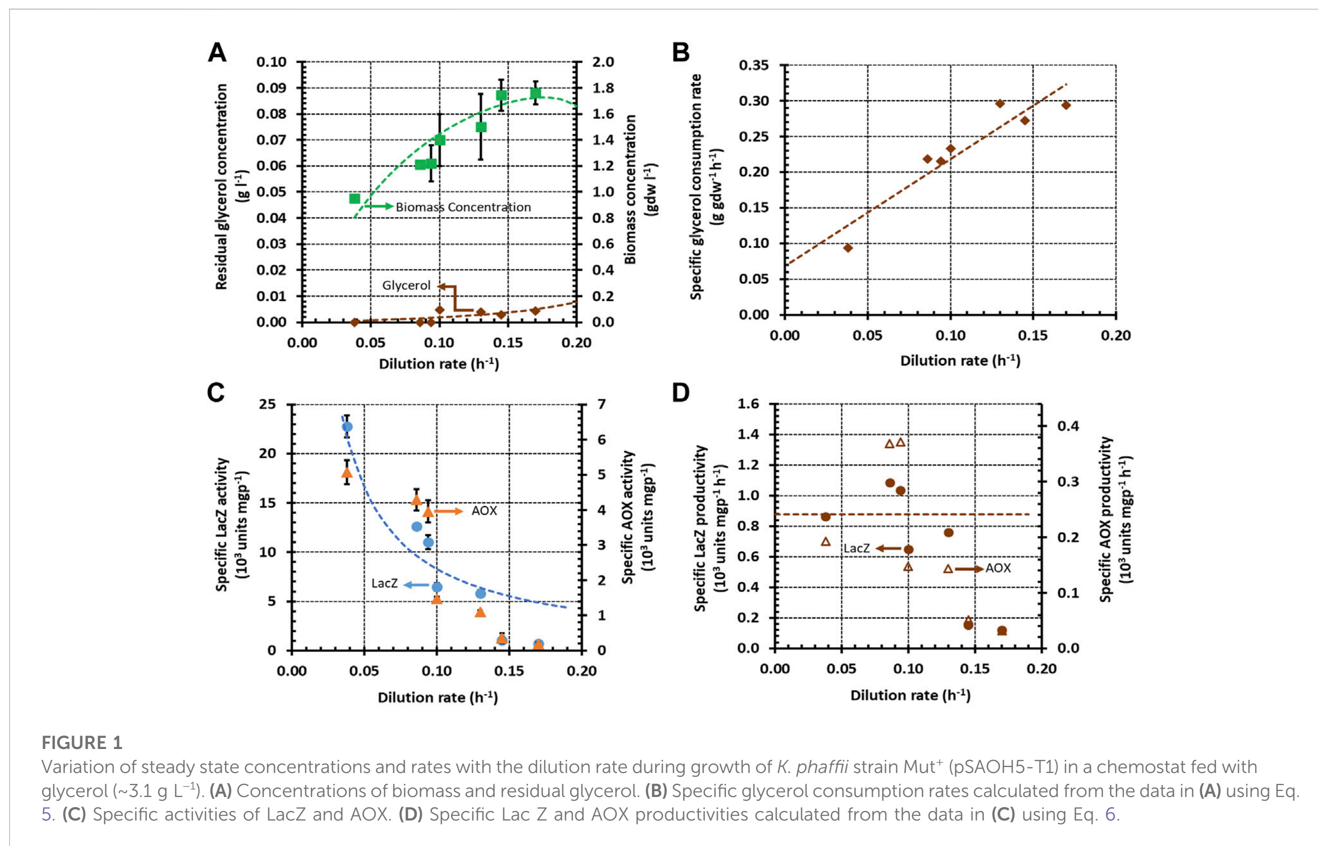

FIGURE 1
Variation of steady state concentrations and rates with the dilution rate during growth of *K. phaffii* strain Mut⁺ (pSAOH5-T1) in a chemostat fed with glycerol (~3.1 g L^{-1}). **(A)** Concentrations of biomass and residual glycerol. **(B)** Specific glycerol consumption rates calculated from the data in **(A)** using Eq. 5. **(C)** Specific activities of LacZ and AOX. **(D)** Specific Lac Z and AOX productivities calculated from the data in **(C)** using Eq. 6.

concentration profile in continuous cultures (Egli et al., 1986; Noel and Narang, 2009) (Supplementary Figure S1A). In the *dual-limited* regime, which extends up to dilution rates approximately equal to the μ_m for the unpreferred substrate, both substrates limit growth, because their residual concentrations s_i are in the order of their saturation constants $K_{s,i}$ ($s_i \sim K_{s,i}$), and therefore, both substrates are completely consumed ($s_i \ll s_{f,i}$). Beyond the dual-limited regime, only the preferred substrate limits growth because the residual concentration of the unpreferred substrate is well above its saturation constant. At the intermediate D, corresponding to the *transition* regime, the preferred substrate is still consumed completely, but the unpreferred substrate is only partially consumed. Beyond the transition regime, the unpreferred substrate is not consumed at all.

When methanol + glycerol and methanol + sorbitol were fed to a continuous culture, the variation of the substrate concentrations with D was consistent with the characteristic pattern described above. In the dual-limited regime, both substrates were completely consumed — up to $D = 0.08\ h^{-1} \approx 0.11\ h^{-1} = \mu_m|_{\text{methanol}}$ (Singh and Narang, 2020) in Figure 2A and $D = 0.03\ h^{-1} = \mu_m|_{\text{sorbitol}}$ in Figure 3A. In the transition regime, the unpreferred substrate was partially consumed up to dilution rates well above its μ_m — up to $D = 0.2\ h^{-1} \approx 2 \times \mu_m|_{\text{methanol}}$ in Figure 2A, and up to $D = 0.08\ h^{-1} \approx 3 \times \mu_m|_{\text{sorbitol}}$ in Figure 3A.

During single-substrate growth, the specific substrate consumption rate usually increases linearly with D up to washout (Pirt, 1965), but during mixed-substrate growth, the specific substrate consumption rates increase linearly with D only in the dual-limited regime (Egli et al., 1986; Noel and Narang, 2009) (Supplementary Figure S1B). The dashed lines in Figures 2B, 3B show that during growth on methanol + glycerol and methanol + sorbitol, the specific methanol consumption rate is indeed proportional to D up to $D = 0.08\ h^{-1}$ and $D = 0.03\ h^{-1}$, respectively. Beyond the respective dual-limited regimes, the specific methanol consumption rates change non-linearly (Supplementary Figure S1B). In the case of methanol + glycerol, the specific methanol consumption rate decreases non-linearly beyond $D = 0.08\ h^{-1}$ due to repression of methanol consumption

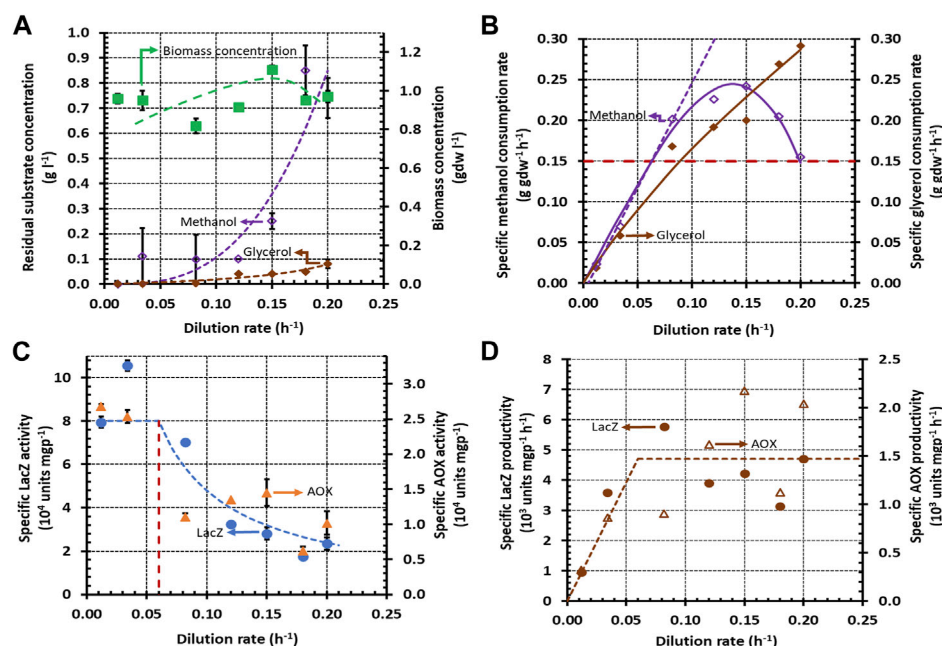

FIGURE 2
Variation of steady state concentrations with the dilution rate during growth of *K. phaffii* strain Mut⁺ (pSAOH5-T1) in a chemostat fed with a mixture of glycerol (~1.5 g L^{-1}) and methanol (~1.6 g L^{-1}). **(A)** Concentrations of biomass, residual glycerol, and residual methanol **(B)** Specific methanol and glycerol consumption rates calculated from the data in **(A)** using Eq. 5. The dashed line passing through the origin shows the linear increase of the specific methanol consumption rate in the dual-limited regime. The horizontal dashed line shows the threshold specific methanol consumption rate of 0.15 g gdw^{-1} h^{-1}. **(C)** Specific activities of LacZ and AOX. **(D)** Specific LacZ and AOX productivities calculated from the data in **(C)** using Eq. 6.

by glycerol (Figure 2B); in the case of methanol + sorbitol, the specific methanol consumption rate increases non-linearly beyond $D = 0.03$ h^{-1} due to the enhanced methanol consumption that occurs to compensate for repression of sorbitol consumption by methanol (Figure 3B). Using Egli's model for dual-limited growth (Egli et al., 1993), we chose feed concentrations such that when growth on both the mixtures is dual-limited ($D \leq 0.03$ h^{-1}), the specific methanol consumption rates of the two mixtures are not only proportional to D, but also *equal* in magnitude. The specific methanol consumption rates of the two mixtures start diverging beyond $D = 0.03\,h^{-1}$, but they remain approximately equal up to $D = 0.05\,h^{-1}$ (compare Figures 2B, 3B).

Although it is widely accepted that glycerol is repressing and sorbitol is non-repressing in batch cultures, we found remarkably similar specific LacZ and AOX activities and productivities in continuous cultures fed with methanol + glycerol and methanol + sorbitol. At low dilution rates ($D \leq 0.05$ h^{-1}), when both mixtures support equal specific methanol consumption rates, the specific LacZ and AOX activities on both mixtures are also equal (Figures 2C, 3C), and hence, their specific LacZ and AOX productivities are also the same (Figures 2D, 3D). At high dilution rates ($D \geq 0.05$ h^{-1}), the specific methanol consumption rates of both mixtures change substantially, but the specific LacZ and AOX productivities are relatively insensitive to this change. Indeed, in the case of methanol + glycerol, the specific methanol consumption rate doubles when D increases from $0.05\,h^{-1}$ to $0.12\,h^{-1}$, and decreases 40% when D increases from $0.12\,h^{-1}$ to $0.20\,h^{-1}$. But the specific LacZ and AOX activities decrease inversely with D (Figure 2C), and hence, the specific LacZ and AOX productivities calculated from Eq. 6 are expected to be constant. These specific productivities, which are shown in Figure 2D, are constant but show considerable scatter at $D \geq 0.05$ h^{-1}. This is expected since at large D, multiplication of e_1 by D amplifies the errors in the measurement of e_1. In the case of methanol + sorbitol, the specific methanol consumption rate doubles when D increases from $0.05\,h^{-1}$ to $0.08\,h^{-1}$, but the specific LacZ and AOX productivities increase only 25% (Figure 3D). Furthermore, the constant maximum specific LacZ and AOX productivities of 4000–6000 units mgp^{-1} h^{-1} and 1200–2000 units mgp^{-1} h^{-1}, respectively, are close to the corresponding maximum values observed during growth on methanol + glycerol. Taken together, these data suggest that the specific P_{AOX1} expression rate is a function of (i.e., completely determined by) the specific methanol consumption rate.

The specific P_{AOX1} expression rate is a function of the specific methanol consumption rate

To test this hypothesis, we plotted the specific LacZ and AOX productivities $r_{e,1}$ at various D in Figures 2D, 3D against the corresponding specific methanol consumption rate $r_{s,1}$ in Figures 2B, 3B. This yielded the graph in Figure 4, which shows that at every specific methanol consumption rate, both mixed-substrate cultures have approximately the same specific P_{AOX1} expression rate (measured as either specific LacZ productivity or specific AOX

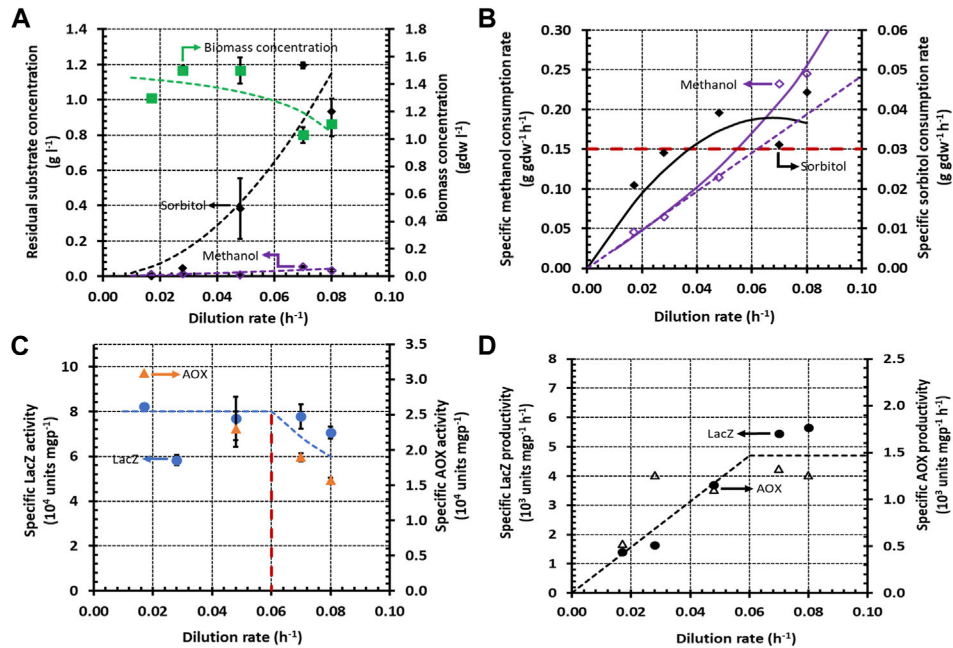

FIGURE 3
Variation of steady state concentrations with the dilution rate during growth of *K. phaffii* strain Mut+ (pSAOH5-T1) in a chemostat fed with a mixture of sorbitol (~1.5 g L−1) and methanol (~3.2 g L−1). **(A)** Concentrations of biomass, residual sorbitol and residual methanol. **(B)** Specific methanol and glycerol consumption rates calculated from the data in **(A)** using Eq. 5. The dashed line passing through the origin shows the linear increase of the specific methanol consumption rate in the dual-limited regime. The horizontal dashed line shows the threshold specific methanol consumption rate of 0.15 g gdw−1 h−1. **(C)** Specific activities of LacZ and AOX. **(D)** Specific LacZ and AOX productivities calculated from the data in **(C)** using Eq. 6.

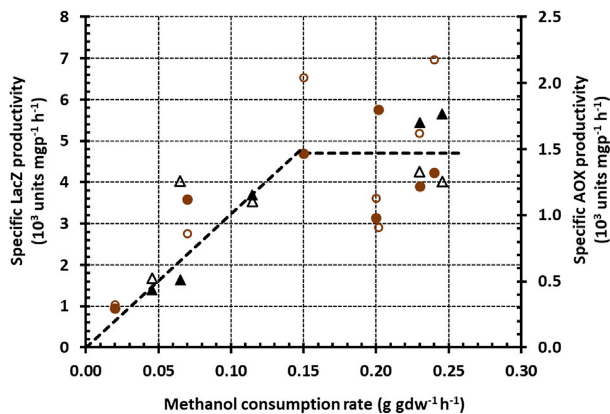

FIGURE 4
Variation of the specific LacZ (closed symbols) and AOX (open symbols) productivities with the specific methanol consumption rate during growth on methanol + glycerol (brown circles) and methanol + sorbitol (black triangles). The graph was obtained by plotting the specific methanol consumption rates in Figures 2B, 3B against the corresponding specific LacZ and AOX productivities in Figures 2D, 3D.

productivity). The specific P_{AOX1} expression rate is therefore completely determined by the specific methanol consumption rate regardless of the type (repressing or non-repressing) of the secondary carbon source. More precisely, the specific P_{AOX1} expression rate, $r_{e,1}$ is proportional to the specific methanol consumption rate, $r_{s,1}$ up to the threshold value ~0.15 g gdw−1 h−1 and remains approximately constant thereafter at the maximum value of ~5 units gdw−1 h−1. Hence, the specific P_{AOX1} expression rates of the mixtures can be approximated by the piecewise linear function

$$r_{e,1} = \begin{cases} V_{e,1}\left(\frac{r_{s,1}}{r_{s,1}^{*}}\right), r_{s,1} \le r_{s,1}^{*} \\ V_{e,1}, r_{s,1} > r_{s,1}^{*} \end{cases} \quad (7)$$

where $V_{e,1}$ denotes the maximum specific P_{AOX1} expression rate, and $r_{s,1}^{*}$ denotes the threshold specific methanol consumption rate beyond which the specific P_{AOX1} expression rate has its maximum value $V_{e,1}$.

Discussion

Our main conclusion is that over the range of dilution rates considered in our work (0.02–0.2 h−1), the P_{AOX1} expression rate is completely determined by the methanol consumption rate regardless of the type of the secondary carbon source. This conclusion may appear to subvert the prevailing consensus according to which the expression rate of a promoter is strongly inhibited in the presence of repressing secondary carbon sources.

However, this conclusion is based on studies with *batch* cultures. We show below that our conclusion is consistent with the *continuous* culture studies reporting the expression of not only the *AOX1* promoter of *K. phaffii* but also the exemplary *lac* promoter of *E. coli*.

Comparison with chemostat studies of P_{AOX1} expression by *K. phaffii*

Jungo *et al.* (Jungo et al., 2007a; Jungo et al., 2007b) performed their mixed-substrate studies by fixing D, $s_{f,1} + s_{f,2}$ and increasing the fraction of methanol in the feed $\sigma_1 = s_{f,1}/(s_{f,1} + s_{f,2})$ at a slow linear rate aimed at maintaining quasi-steady state. They found that as, σ_1 increased:

a) The residual methanol remained negligibly small, and the biomass concentration decreased linearly.
b) The specific avidin expression rate increased hyperbolically until it reached a maximum, which was essentially the same for both mixtures.

It follows from a) that the specific methanol consumption rate, which is approximately equal to $D(s_{f,1} + s_{f,2})\sigma_1/x$, increased throughout their experiment. But then b) implies that, as the specific methanol consumption rate increased, the specific avidin expression rate of both mixed-substrate cultures reached essentially the *same* maximum (cf. Figure 4).

Berrios and co-workers compared the methanol consumption and ROL production rates of the Mut$^+$ strain at two different temperatures (22°C and 30°C) during growth on methanol, methanol + glycerol, and methanol + sorbitol (Berrios et al., 2017). These experiments were done in chemostats operated at D = 0.03 h^{-1}, and in the case of mixed-substrate experiments, fed with two feed compositions (40 and 70 C-mole % methanol). They found that "Sorbitol-based cultures led to a higher q_p than both glycerol-based and control cultures at most studied conditions." But closer inspection shows that in all their experiments, the specific expression rates were 0.8–0.9 units gdw^{-1} h^{-1}, which is close to the maximum specific expression rate of 1-1.1 unit gdw^{-1} h^{-1}.

Comparison with chemostat studies of expression by *lac* promoter of *E. coli*

Analogous results have also been obtained in studies of *lac* expression in *E. coli.* Indeed, batch experiments with mixtures of lactose + glycerol, lactose + glucose, and lactose + glucose-6-phophate show that glycerol is non-repressing, whereas glucose and glucose-6-phosphate are repressing (Magasanik, 1970). However, when chemostat experiments were performed with these three mixtures (Smith and Atkinson, 1980), they yielded the *same* steady state specific β-galactosidase (LacZ) activity at all $D \lesssim 0.5$ h^{-1} (Supplementary Figure S2). Furthermore, when the steady state specific LacZ activities at various D were plotted against the corresponding specific lactose consumption rates at the same D, the data for all three mixtures collapsed into a single line (Supplementary Figure S3). This led the authors to conclude that the steady state specific LacZ activity was "an apparently linear function of the rate of lactose utilization independent of the rate of metabolism of substrates other than lactose which are being concurrently utilized." But then it follows from Eq. 6 that the steady state specific LacZ productivity is also completely determined by the specific lactose consumption rate regardless of the type (repressing or non-repressing) of the secondary carbon source (Supplementary Figure S4).

In conclusion, the specific P_{AOX1} expression rate of *K. phaffii* appears to be completely determined by the specific methanol consumption rate regardless of the type (repressing or non-repressing) of the secondary carbon source. Analysis of the literature shows that the specific expression rate of the *lac* operon of *E. coli* is also completely determined by the specific lactose consumption rate regardless of the type of secondary carbon source. It would be interesting to explore if similar results are obtained for other microorganisms and substrate mixtures.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Author contributions

AS and AN conceived and designed the research. AS conducted the experiments. AS and AN analysed the data and wrote the manuscript. All authors read and approved the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2023.1123703/full#supplementary-material

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