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Wenhui Zhang

Karen J. Hywood Potter

Bradley A. Plantz

Vicki L. Schlegel
University of Nebraska-Lincoln, vschlegel3@unl.edu

Leonard A. Smith

See next page for additional authors

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Authors
Wenhui Zhang, Karen J. Hywood Potter, Bradley A. Plantz, Vicki L. Schlegel, Leonard A. Smith, and Michael M. Meagher

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ORIGINAL PAPER

Wenhui Zhang · Karen J. Hywood Potter
Bradley A. Plantz · Vicki L. Schlegel
Leonard A. Smith · Michael M. Meagher

Pichia pastoris fermentation with mixed-feeds of glycerol and methanol: growth kinetics and production improvement

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Abstract Fed-batch fermentation of a methanol utilization plus (Mut^+) *Pichia pastoris* strain typically has a growth phase followed by a production phase (induction phase). In the growth phase glycerol is usually used as carbon for cell growth while in the production phase methanol serves as both inducer and carbon source for recombinant protein expression. Some researchers employed a mixed glycerol-methanol feeding strategy during the induction phase to improve production, but growth kinetics on glycerol and methanol and the interaction between them were not reported. The objective of this paper is to optimize the mixed feeding strategy based on growth kinetic studies using a Mut^+ *Pichia* strain, which expresses the heavy-chain fragment C of botulinum neurotoxin serotype C [BoNT/C(Hc)] intracellularly, as a model system. Growth models on glycerol and methanol that describe the relationship between specific growth rate (μ) and specific glycerol/methanol consumption rate (v_{gly}, v_{MeOH}) were established. A mixed feeding strategy with desired $\mu_{gly}/\mu_{MeOH} = 1, 2, 3, 4$ (desired μ_{MeOH} set at 0.015 h^{-1}) was employed to study growth interactions and their effect on production. The results show that the optimal desired μ_{gly}/μ_{MeOH} is around 2 for obtaining the highest BoNT/C(Hc) protein content in cells: about 3 mg/g wet cells.

W. Zhang (✉) · K. J. Hywood Potter · B. A. Plantz
M. M. Meagher
Department of Chemical Engineering,
University of Nebraska-Lincoln, 207M Othmer Hall,
Lincoln, NE 68583-0919, USA
E-mail: wzhang1@unl.edu
Tel.: +1-402-4729301
Fax: +1-402-4726989

V. L. Schlegel
Department of Food Science and Technology,
University of Nebraska, Lincoln, NE 68588, USA

L. A. Smith
United States Army Medical Research Institute
of Infectious Diseases, Frederick, MD 21702, USA

Keywords *Pichia pastoris* · Fed-batch fermentation · Mixed feed · Growth model · Botulinum neurotoxin

Introduction

In the genome of the methylotrophic yeast *Pichia pastoris*, there are two copies of the alcohol oxidase (AOX) gene, designated *AOX1* and *AOX2*. These genes enable the cells to assimilate methanol as their sole carbon and energy source. The *AOX1* promoter regulates 85% of AOX production while the *AOX2* promoter is less active [9]. *Pichia* expression systems use the *AOX1* promoter to drive heterologous protein expression with methanol as the inducer. Through gene disruption [7], the "*AOX1* promoter-interesting gene" expression cassette is inserted in the genome [9, 10, 16]. Depending on the locus of insertion, two different phenotypes of *Pichia* are generated: methanol utilization plus (Mut^+) or methanol utilization slow (Mut^s). The former contains both *AOX1* and *AOX2*, the latter only *AOX2*. For Mut^s strains, due to their slow utilization of methanol, a mixed feed of glycerol and methanol is commonly employed in the fermentation induction phase; glycerol functions as an efficient substrate for cell growth and target protein production while methanol functions as an inducer. With this strategy, various proteins have been successfully expressed in either fed-batch or continuous operation mode by Mut^s strains [1, 2, 14, 19]. In all of these studies, the optimization of mixed feeding strategy was based on arbitrary ratios of the two substrates in the feed solution. Growth kinetics on glycerol and methanol, and the interaction between them were not studied.

For Mut^+ strains of *Pichia*, due to efficient utilization of methanol, a typical fed-batch fermentation strategy is to feed methanol alone as both inducer and carbon and energy source in the induction phase. We developed an exponential feeding strategy based on a growth model describing the relationship between

specific growth rate (μ) and specific methanol consumption rate (v_{MeOH}) [23]. This makes it possible to maximize production based on an optimal μ. A basic protocol book, "*Pichia fermentation process guidelines*", which provides a methanol feeding strategy with stepwise changing feed rate, is also available from Invitrogen (San Diego, Calif.).

Mixed feeding strategies in Mut$^+$ *Pichia* fermentations similar to those for Mut^s strains have been reported. Katakura et al. [13] determined that product formation was dramatically improved by simultaneously feeding glycerol at a rate of 5 ml l^{-1} h^{-1} while maintaining 0.55% (v/v) residual methanol in the induction phase. McGrew et al. [15] employed mixed feeds at a glycerol:methanol ratio of 1:1 and approximately doubled growth and CD40 ligand expression levels compared to feeding methanol alone. In contrast, Hellwig et al. [12] found that, while maintaining a 0.5% methanol concentration in the induction phase, supplementary feeding of glycerol strongly inhibited production of an scFv antibody fragment, and expression was almost completely inhibited when the specific glycerol feed rate (F_{gly}) was higher than 6 mg mg-wet cell weight (WCW)$^{-1}$ h^{-1}. Even at F_{gly} below 6 mg mg-WCW^{-1} h^{-1}, the expression level was only half that found in the fermentation with methanol feed alone. These attempts at running a mixed feeding strategy in Mut$^+$ *Pichia* fermentations did not further investigate the interaction of cell growth on the two substrates. The objective of this paper is to study mixed feeding strategies based on cell growth kinetics to determine the potential of improving product formation. We used a GS115 Mut$^+$ *Pichia* strain that intracellularly expresses the heavy-chain fragment C of botulinum neurotoxin serotype C [BoNT/C(Hc)] as a model system. When growing on methanol, the BoNT/C(Hc) strain showed a maximum specific growth rate (μ_m) of around 0.02 h^{-1} (as shown later), distinguishing it from a value 0.07 h^{-1} obtained for another Mut$^+$ *Pichia* strain expressing a similar protein, BoNT/A(Hc) [23] and a Mut^s strain we measured at 0.008 h^{-1} (data not shown). Therefore, choosing a BoNT/C(Hc) strain for this study could be representative for both Mut^s and Mut$^+$ *Pichia* expression systems. The strain was constructed at the United States Army Medical Research Institute of Infectious Diseases (USAMRIID). BoNT/C(Hc) is one of the seven BoNT(Hc) serotypes, designated A–G, corresponding to seven different strains of *Clostridium botulinum*. The recombinant BoNT(Hc) are nontoxic 50 kDa fragments that elicit significant protective immunity in mice and are candidate vaccines against botulinum neurotoxin [3, 5].

Materials and methods

Fermentation conditions

A 1-l shake flask with 300 ml buffered minimal glycerol yeast extract medium (BMGY, containing 1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 4×10^{-5}% biotin, 1% glycerol, 100 mM potassium phosphate, pH 6.0) was inoculated with 1 ml stock seed. The cultivation lasted for 20–24 h at 30°C and 300 rpm to reach an optical density (at 600 nm) of 10–20. The entire 300 ml propagated culture was used to inoculate a 5-l fermentor (New Brunswick Scientific, Edison, N.J.) containing 2 l basal salts medium (BSM) and 8.7 ml PTM_1 trace salts. One liter BSM consists of 26.7 ml 85% H_3PO_4, 0.93 g $CaSO_4$, 18.2 g K_2SO_4, 14.9 g $MgSO_4{\cdot}7H_2O$, 4.13 g KOH, and 40.0 g glycerol. One liter PTM_1 (filter-sterilized) consists of 6.0 g $CuSO_4{\cdot}5H_2O$, 0.08 g NaI, 3.0 g $MnSO_4{\cdot}H_2O$, 0.2 g $Na_2MoO_4{\cdot}2H_2O$, 0.02 g H_3BO_3, 0.5 g $CoCl_2$, 20.0 g $ZnCl_2$, 65.0 g $FeSO_4{\cdot}7H_2O$, 0.2 g biotin and 5.0 ml H_2SO_4. The fermentation was run in fed-batch mode at 30°C, and pH was maintained at 5.0 using undiluted (28%) ammonium hydroxide. Dissolved oxygen (DO) was maintained above 20% saturation by adjusting agitation rate and pure oxygen supply.

When the initial glycerol (40 g/l) in batch phase was depleted, as indicated by an abrupt increase in DO reading, a 63% (w/v) glycerol solution containing 1.2% (v/v) PTM_1 was fed at a feed rate of 12 ml h^{-1} l-broth^{-1} for 1 h (growth was limited by the feed rate and no glycerol accumulation occurred). Methanol (4 ml) was then injected into the fermentor and, simultaneously, the glycerol feed rate was programmed to decrease linearly from 12 ml h^{-1} l^{-1} to 0 over a period of 3 h. This 3-h period was considered a transition phase that is important for cells to adapt to the methanol efficiently and completely [23]. Following the transition phase, the production phase started, in which 100% methanol containing 1.2% (v/v) PTM_1 and 0.05% (w/v) antifoam (KFO 673, KABO Chemicals, Cheyenne, Wyo.) was fed under control of a methanol sensor (MC-168 Methanol Monitor and Controller, PTI Instruments, Kathleen, Ga.; the sensing material in the MC-168 is TGS822 alcohol sensor from Figaro USA, Glenview, Ill.), or at a programmed feed rate based on a desired growth rate. Five or six time-course samples, taken every 4–6 h, were removed during the production phase.

To determine the growth model on a glycerol phase, a 63% (w/v) glycerol solution containing 1.2% (v/v) PTM_1 was fed at a programmed feed rate based on a desired growth rate (glycerol feeding strategy as shown later), after the initial glycerol batch phase. Four to six time-course samples, taken every 3–5 h, were removed during the glycerol fed-batch phase.

Cell density measurement

Cell density was expressed as grams WCW per liter broth, which was obtained by centrifuging the samples at 2,000 *g* for 10 min. All kinetic calculations were based on WCW. The methanol concentration in samples was measured by gas chromatography (GC-17A, Shimadzu, Columbia, Md.) with isopropyl alcohol as an internal standard.

BoNT/C(Hc) analysis

BoNT/C(Hc) protein was released from cells through bead breaking, and quantified by western blot analysis. Cell paste samples of 0.2–0.5 g were suspended in 10 ml washing buffer (145 mM NaCl, 31.5 mM sodium acetate, 18.5 mM acetic acid, pH 5.0), then spun at 2,000 *g* for 10 min to obtain washed cell pellets. The cell pellets were resuspended to a density of 50 g-WCW/l in lysis buffer, which contains 2.5 g/l 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 5 mM EDTA, 500 mM NaCl, 50 mM NaH_2PO_4, 1 mM phenylmethanesulfonyl fluoride (PMSF), and pH adjusted to 7.5. Aliquots (1 ml) of the cell suspension were mixed with approximately 2.2 g zirconia/silica beads (Biospec, Bartlesville, OK) in 2.0 ml screw cap tubes followed by disruption at 4°C with a vibrating disrupter (Mini-BeadBeater-8, Biospec) for eight cycles (1 min vibrating and 4 min resting/cycle). The lysate/bead mixture was centrifuged until the supernatant was clear.

The acquired supernatant was diluted 3-fold with lysis buffer, and 90 μl diluted supernatant was mixed with 30 μl 4× concentrated Tris-glycerine SDS sample buffer (252 mM Tris-HCl, 40% glycerol, 8% SDS, 0.01% bromophenol blue, pH 6.8). The mixture

was heated at 90°C for 5–10 min, then 35-μl aliquots were loaded onto a 10-well 4–20% Tris-glycine SDS-PAGE gel (Novex Pre-Cast gel, Invitrogen, San Diego, Calif.). Each sample was loaded in duplicate. A standard sample containing 703.13 ng BoNT/C(Hc) was loaded on each gel. After running the SDS-PAGE (150 V, 1.8 h, running buffer: SepraBuff TRIS GLY SDS running buffer, OWL Seperation Systems, Woburn, Mass.), the gel was soaked in a transfer buffer (SepraBuff TRIS GLY running and blotting buffer) for 5–10 min; proteins were then transferred from the gel to a polyvinylidene fluoride (PVDF) membrane (soaked in methanol before use) using a semi-dry transfer cell (Trans-Blot SD, Bio-Rad, Hercules, Calif.; 12 V, 1 h). Following transfer, the PVDF membrane was soaked in blocker solution (5% blotting grade dry milk in 100 ml TBS buffer: 25 mM Tris base, 140 mM NaCl, 2.5 mM KCl) for 1 h. The membrane was incubated with the primary antibody solution for 16 h, then rinsed 3×10 min in TBS buffer, and soaked in secondary antibody solution for 2–4 h. The final rinse was 3×10 min in TBS buffer. Protein on the membrane was visualized by chemiluminescence using ECL + Plus (Lumigen, Southfield, Mich.) with a 5 min incubation (in the dark), followed by exposure of two films (Hyperfilm ECL, Amersham Pharmacia, Piscataway, N.J.) each with a different exposure time (2–5 min). The films were developed (Kodak GBX fixer and developer) to reveal the bands of BoNT/C(Hc) protein. Band intensities were obtained by scanning the films (HP ScanJet 6300C scanner) and digitizing the bands (UN-SCAN-IT, Automated Digitizing System, Version 5.1, Silk Scientific Corporation, Orem, Utah). BoNT/C(Hc) in each band was quantified from a standard curve that showed the relationship between band intensity and protein amount.

Results and discussion

Growth kinetics on glycerol

In the batch phase, with an initial glycerol concentration of 40 g/l, cells were grown without limitation of nutrients. The maximum growth rate on glycerol, $\mu_{gly,m}$, and maximum glycerol specific consumption rate, $v_{gly,m}$, were determined as $\mu_{gly,m} = 0.177\ h^{-1}$ and $v_{gly,m} = 0.0688\ g\ h^{-1}\ g\text{-}WCW^{-1}$. Knowing $\mu_{gly,m}$ and $v_{gly,m}$, the glycerol feed rate, F_{gly}, to run a fed-batch process with the desired $\mu_{gly,d}$ ($\leq \mu_{gly,m}$) can be estimated as in [23]:

$$F_{gly} = v_{gly} X_0 V_0 e^{\mu_{gly,d} t} = \frac{\mu_{gly,d} v_{gly,m} X_0 V_0 e^{\mu_{gly,d} t}}{\mu_{gly,m}} \quad (1)$$

where X_0 and V_0 are the cell density and broth volume at the beginning of the glycerol fed-batch phase, and t the feed time of glycerol. Fed-batch runs with different $\mu_{gly,d}$ from 0 to $\mu_{gly,m}$ were conducted using the feeding strategy in Eq. 1. The actual μ_{gly} (which could differ from $\mu_{gly,d}$) and v_{gly}, was determined from each run. The method used to calculate the specific rates (as well as all other kinetics) was referred to in a previous publication [23]. Figure 1 shows the linear dependence of v_{gly} on μ_{gly}, and the resulting equation:

$$v_{gly} = 0.503\mu_{gly} + 0.0065 \quad (2)$$

With the fed-batch growth model in Eq. 2, estimation of F_{gly} by Eq. 1) was corrected to realize a growth rate approaching $\mu_{gly,d}$, namely:

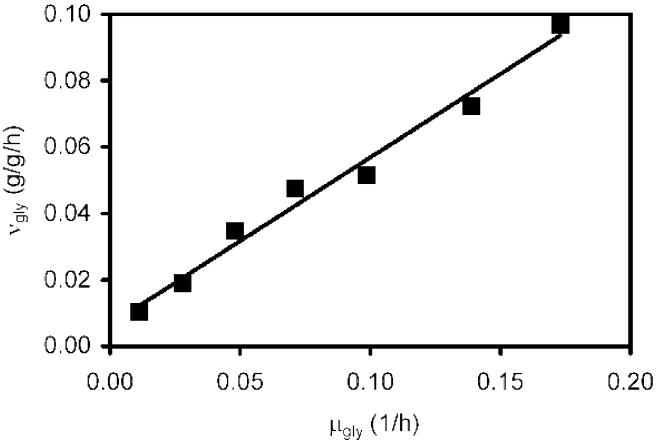

Fig. 1 Dependence of glycerol specific consumption rate (v_{gly}) on specific growth rate (μ_{gly}) in glycerol fed-batch growth

$$F_{gly} = (0.503\mu_{gly,d} + 0.0065) X_0 V_0 e^{\mu_{gly,d} t} \quad (3)$$

As shown later, the feeding strategy of Eq. 3 will be applied to the design of fed-batch fermentations with mixed feeds of glycerol and methanol assuming the coexisting methanol feed has no effects on the kinetics of glycerol consumption.

The catabolic pathway of glycerol in a methylotrophic yeast such as *Pichia*, involves passive diffusion across the membrane, phosphorylation by a glycerol kinase, and oxidation by a mitochondrial glycerol phosphate ubiquinone oxireductase [11]. Glycerol enters glycolysis after its conversion to glyceraldehyde 3-phosphate, and requires respiration to oxidize NADH in order to serve as an energy source. The glycerol catabolic process is independent of the methanol metabolism pathways described elsewhere [18]. Therefore, although the growth model on glycerol, Eq. 2, was obtained with the BoNT/C(Hc) strain employed in this research, the model can be applied to the design of fed-batch growth on glycerol for all other GS115-derived *Pichia* clones, whether they are Mut^+ or Mut^s.

Growth kinetics on methanol

P. pastoris cannot tolerate a high methanol concentration in the fermentation due to accumulation of formaldehyde and hydrogen peroxide inside the cells, both of which are oxidized products of methanol by AOX and toxic to the cells [6, 8, 21]. As discovered in our previous work [23], methanol levels above 3.65 g/l (calculated from the growth model) start to show growth inhibition. Therefore, unlike growth on glycerol, it is impossible to measure the maximum growth rate ($\mu_{MeOH,m}$) by growing cells in batch mode with a high methanol level. To reveal the $\mu_{MeOH,m}$, as well as the corresponding maximum methanol specific consumption rate ($v_{MeOH,m}$), we ran the fed-batch fermentation employing a methanol sensor to control methanol feeding to maintain the

methanol level at 2–4 g/l. A proportional, integral and derivative (PID) control mode was applied for the methanol control system [24]. Figure 2 shows the time-course of total cell growth (XV) and the methanol level (S) controlled by the sensor. Exponential growth was observed within 50 h of methanol feeding time. The $\mu_{MeOH,m}$ and $v_{MeOH,m}$ were 0.02 h^{-1} and 0.028 g h^{-1} g WCW^{-1}, respectively. The $\mu_{MeOH,m}$ is much lower than that of a typical Mut^+ *Pichia* strain, i.e., 0.07 h^{-1} [24], but higher than a Mut^s strain, 0.008 h^{-1} (data not shown). This suggests that methanol metabolism of the BoNT/C(Hc) *Pichia* strain was affected by either expression of the heterologous protein or the gene insertion event. A similar phenomenon was also observed in an *Escherichia coli* expression system in which a recombinant protein changed host strain bioactivities [22].

Based on the obtained $\mu_{MeOH,m}$ and $v_{MeOH,m}$, we conducted fed-batch fermentations with different desired growth rates, $\mu_{MeOH,d}$ ($\leq \mu_{MeOH,m}$), by feeding methanol at a feed rate, F_{MeOH}, estimated as in [23]:

$$F_{MeOH} = v_{MeOH} X_0 V_0 e^{\mu_{MeOH,d} t} = \frac{\mu_{MeOH,d} v_{MeOH,m} X_0 V_0 e^{\mu_{MeOH,d} t}}{\mu_{MeOH,m}} \quad (4)$$

where X_0 and V_0 are the cell density and broth volume at the beginning of the methanol fed-batch phase, and t the feed time of methanol. Similar to the limited fed-batch growth on glycerol, an actual μ_{MeOH} and v_{MeOH} were obtained from each run, and the linear dependence of v_{MeOH} on μ_{MeOH} is shown in Fig. 3. The linear relationships are expressed as:

$$v_{MeOH} = 0.766\mu_{MeOH} + 0.0128 \quad (5)$$

Equation 5 includes the maintenance coefficient, 0.0128 g-MeOH h^{-1} g-WCW^{-1}, and was substituted into Eq. 4, to give Eq. 6:

$$F_{MeOH} = (0.766\mu_{MeOH,d} + 0.0128) X_0 V_0 e^{\mu_{MeOH,d} t} \quad (6)$$

Mixed feed and production improvement

The BoNT/C(Hc) content in the cells (α) reached a maximum after 10 h of methanol feeding, and remained constant during the remainder of the exponential growth phase, similar to the intracellular production of BoNT/A(Hc) [23]. The average α during the stable period (defined as quasi-steady state) represented the production level at a corresponding μ_{MeOH}. The effect of μ_{MeOH} on α using only methanol during fed-batch fermentation is presented in Fig. 4. The optimum μ_{MeOH} was 0.015 h^{-1} and achieved $\alpha = 2$ mg/g. This is similar to that observed for BoNT/A(Hc) production [23] in which the optimal μ_{MeOH} was 0.0267 h^{-1} rather than the $\mu_{MeOH,m}$ (0.0709 h^{-1}).

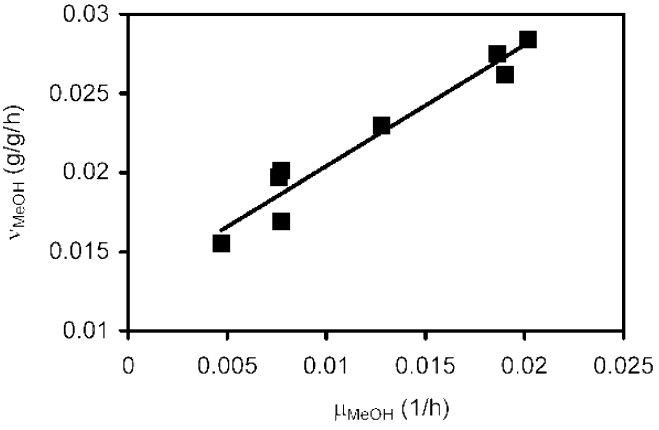

Fig. 3 Dependence of methanol specific consumption rates (v_{MeOH}) on specific growth rate (μ_{MeOH}) in methanol fed-batch growth

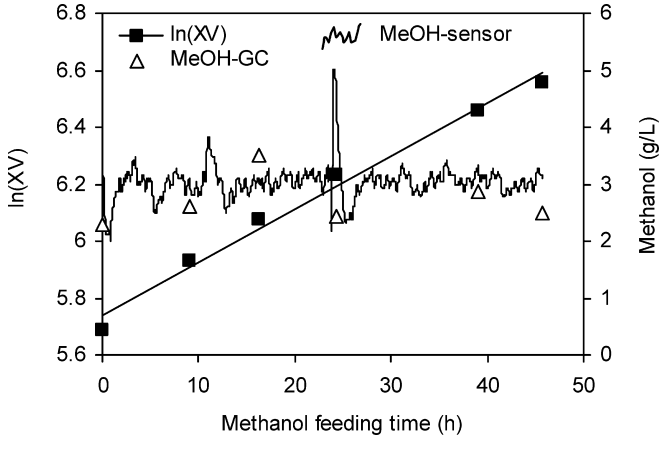

Fig. 2 Time-course of total grown cells and controlled methanol level in the methanol fed-batch phase. *MeOH-sensor* Methanol online read value from the methanol sensor, *MeOH-GC* methanol concentration in the samples analyzed by gas chromatography

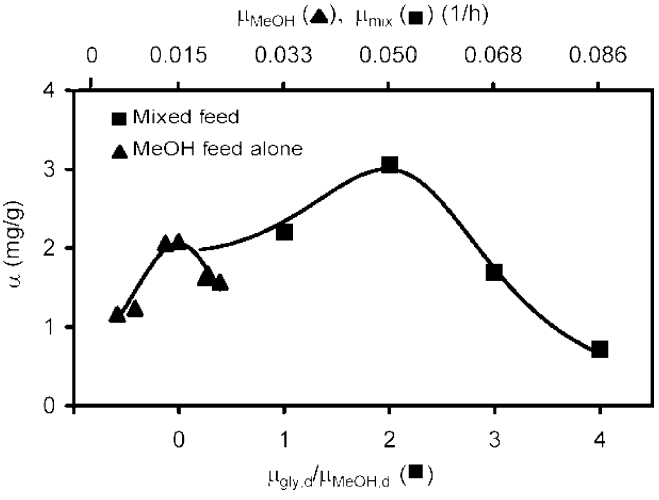

Fig. 4 Heavy-chain fragment C of botulinum neurotoxin serotype C [BoNT/C(Hc)] content in cells (α) under methanol feed alone and mixed feed

The detailed mechanism explaining why μ affects α remains unknown, but the imbalance of energy supply for growth and production is believed to be one of the reasons [4, 17]. Based on this supposition, and considering the slow methanol assimilation in this strain, we proposed a mixed feeding strategy, namely, feeding glycerol simultaneously in the methanol fed-batch phase, to explore the potential for improving production. As we discussed, the mixed feeding strategy is generally employed for Mut^s *Pichia* fermentations in view of their slow utilization of methanol, and has also been applied in Mut$^+$ strains by several researchers. However, all these studies relied on arbitrary combinations of the two substrates (glycerol and methanol) for optimizing the mixed feed design. Here we investigated the strategy based on the growth kinetics. Specifically, we optimized the growth rate ratio of μ_{gly}:μ_{MeOH} rather than the quantity ratio of glycerol:methanol in the mixed feed. μ_{gly} and μ_{MeOH} are the growth rate contributed by glycerol and methanol, respectively, and the total growth rate on a mixed feed, μ_{mix}, expressed as:

$$\mu_{mix} = \mu_{gly} + \mu_{MeOH} \quad (7)$$

We ran a mixed feeding strategy with desired μ_{gly}:μ_{MeOH} = 1, 2, 3, 4 to study the growth interaction of the two substrates and its effect on production. With growth on methanol feed alone, μ_{MeOH} = 0.015 h^{-1} was the optimal growth rate for maximum α. Accordingly, we fixed the desired growth rate on methanol ($\mu_{MeOH,d}$) at 0.015 h^{-1} while varying the desired growth rate on glycerol ($\mu_{gly,d}$) for the mixed feed design. Based on Eqs. 3 and 6, the glycerol and methanol feed rate were given as:

$$F_{gly} = (0.503\mu_{gly,d} + 0.0065)X_0V_0e^{\mu_{mix,d}t} \quad (8)$$

$$F_{MeOH} = (0.766\mu_{MeOH,d} + 0.0128)X_0V_0e^{\mu_{mix,d}t} \quad (9)$$

where $\mu_{mix,d}$ was calculated as $\mu_{MeOH,d}$ + $\mu_{gly,d}$ according to Eq. 7, and t the feed time of glycerol and methanol. Figure 5 shows the actual μ_{mix} achieved from the runs performing the feeding strategy of Eqs. 8 and 9 with $\mu_{MeOH,d}$ = 0.015 h^{-1} and various $\mu_{gly,d}$. It was found that μ_{mix} was slightly higher than $\mu_{mix,d}$ and had the following relationship with $\mu_{gly,d}$:

$$\mu_{mix} = 1.18\mu_{gly,d} + 0.015 \quad (10)$$

Compared with Eq. 7, and noting $\mu_{MeOH,d}$ = 0.015 h^{-1}, Eq. 10 shows that the total growth was promoted in the presence of glycerol feeding. It is known that excess glycerol inhibits the *AOX* promoter [20]. We obtained the opposite result due to the fact that the glycerol feed rate we ran only supported growth below 0.06 h^{-1}, which was far from the maximum μ on glycerol (0.177 h^{-1}). Thus, we concluded that during growth on a mixed feed with $\mu_{gly,d} \leq$ 0.06 h, i.e., $\mu_{gly,d}$:$\mu_{MeOH,d} \leq 4$ for this strain, the supplementary feeding of glycerol enhanced the overall growth rather than functioned as a repressor. This observation indicates that running a mixed feed in Mut$^+$ *Pichia* fermentations is feasible without causing growth inhibition by glycerol when the feeding strategy is properly designed. The production level under the mixed feed design can be examined to discover the optimal feeding strategy.

Figure 4 shows the production levels obtained under the mixed feed strategy with various $\mu_{gly,d}/\mu_{MeOH,d}$ while $\mu_{MeOH,d}$ was set to 0.015 h^{-1}, as well as a comparison with methanol feed alone. It was found that $\mu_{gly,d}/\mu_{MeOH,d}$ = 2, which corresponded to an obtained μ_{mix} of 0.05 h^{-1}, delivered the highest α, and the production was not inhibited by the supplementary glycerol feeding until a feeding strategy with $\mu_{gly,d}/\mu_{MeOH,d}$ > 3 was run. Substituting the optimal $\mu_{gly,d}$ = 0.03 h^{-1} and $\mu_{MeOH,d}$ = 0.015 h^{-1} into Eqs. 8 and 9, the optimal feed rate ratio of F_{gly}:F_{MeOH} was simply derived to be 0.889. This discovery demonstrated that the mixed feeding strategy based on growth kinetics as in Eqs. 2 and 5 can be optimized to maximize production. The optimal strategy can be applied to any cell densities as a result of the direct association with growth rate instead of the arbitrary combinations of the two substrates that was used in previous researches. Since growth models were developed on both glycerol (Eq. 2) and methanol (Eq. 5), a thorough simulation for the optimal process can be made in the same way as for optimization of BoNT/A(Hc) production [23]. It is predicted that the methods developed in this paper can be applied to mixed feed design for both Mut$^+$ and Mut^s *Pichia* fermentations producing a variety of recombinant proteins.

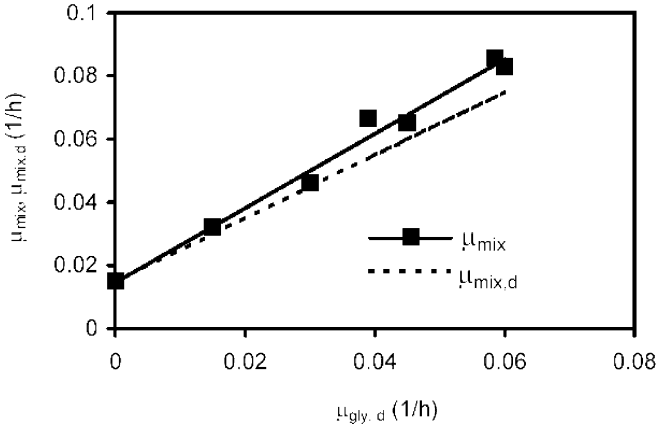

Fig. 5 Actual μ_{mix} achieved from the runs with $\mu_{MeOH,d}$= 0.015 h^{-1} and various $\mu_{gly,d}$. $\mu_{mix,d}$ = $\mu_{MeOH,d}$ + $\mu_{gly,d}$ was also plotted as a *dotted line*

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