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Determination of a Dynamic Feeding Strategy for Recombinant *Pichia pastoris* Strains

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Abstract

The knowledge of certain strain specific parameters of recombinant *P. pastoris* strains is required to be able to set up a feeding regime for fed-batch cultivations. To date, these parameters are commonly determined either by time-consuming and labor-intensive continuous cultivations or by several, consecutive fed-batch cultivations. Here, we describe a fast method based on batch experiments with methanol pulses to extract certain strain characteristic parameters, which are required to set up a dynamic feeding strategy for *P. pastoris* strains based on specific substrate uptake rate (q_s). We further describe in detail the course of actions which have to be taken to obtain the desired dynamics during feeding.

Keywords

Pichia pastoris; Methanol pulses; Specific substrate uptake rate; Dynamic fed-batch strategy

1 Introduction

Recombinant protein production with the methylotrophic yeast *Pichia pastoris* is a key process not only in academic research but also in the biopharmaceutical industry. To date, several of the implemented fermentation strategies for *P. pastoris* are based on the Invitrogen protocol suggesting constant feeding profiles for fed-batch cultivations (<http://tools.invitrogen.com>). Different strategies, like a feed forward regime based on a constant specific growth rate (μ , e.g., [1, 2]), are based on this protocol. However, these strategies do not aim at minimizing substrate consumption or at improving and optimizing the specific productivity.

One of the major goals of each recombinant protein production process is a maximum productivity. Currently, the outcomes regarding a possible interdependency between the specific productivity (q_p) and the specific growth rate (μ) of *P. pastoris* are inconsistent as some studies show that q_p does not relate to μ [1, 3, 4], whereas another study demonstrates growth association [5]. Due to these controversial findings, another parameter than μ , namely, the specific substrate uptake rate (q_s), has been analyzed for its possible correlation with q_p recently [6-8].

However, regardless of which control parameter is chosen to set up a feeding profile for *P. pastoris*, specific parameters of the respective strain have to be determined. This can either

be done by very time-consuming and labor-intensive continuous cultivations [9] or by several, consecutive fed-batch cultivations [10, 11]. However, to meet industrial demands, a fast and easy-to-do characterization of recombinant *P. pastoris* strains to extract bioprocess-relevant strain characteristic parameters for the subsequent set-up of production processes is essential.

Here, we describe a novel, fast method based on batch experiments with methanol pulses to extract a minimal set of strain characteristic parameters, which are required to set up a dynamic feeding strategy for *P. pastoris* strains based on q_s .

2 Materials

Prepare all media and solutions with analytical grade reagents and deionized water.

2.1 Medium for Preculture

Yeast nitrogen base (YNB) medium per L: 1.0 M potassium phosphate buffer (dissolve 118.1 g KH_2PO_4 and 23.0 g K_2HPO_4 in 1,000 mL distilled water, pH 6.0), 3.4 g YNB w/o Amino acids and Ammonium Sulfate, 10 g $(\text{NH}_4)_2\text{SO}_4$, 400 mg biotin, 20 g glucose. Weigh YNB w/o amino acids and ammonium sulfate $(\text{NH}_4)_2\text{SO}_4$, biotin, and glucose in a beaker. Add 100 mL of 1.0 M potassium phosphate buffer (pH 6.0), dissolve by stirring and set with water to 1 L. Filter-sterilize through a 0.2 μm cutoff filter into a sterile flask. If the strain of interest carries a resistance gene, add the respective antibiotics according to the specific resistance marker present in the strain (e.g., Zeocin, Kanamycin) to an appropriate concentration (e.g., 100 $\mu\text{g/mL}$ medium). Store at 4 °C.

2.2 Medium for Batch and Fed-Batch Culture

1. Basal salt medium (BSM) per L: 26.7 mL of 85 % (v/v) phosphoric acid; 1.17 g $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$, 18.2 g K_2SO_4 , 14.9 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 4.13 g KOH, 44 g $\text{C}_6\text{H}_{12}\text{O}_6 \cdot \text{H}_2\text{O}$, 0.3 mL Antifoam. Weigh the chemicals in a beaker, dissolve in around 600 mL of water, and then fill up to 725 mL in a measuring cylinder. Fill the bioreactor with this medium and autoclave.
2. C-source per L: weigh 220 g glucose monohydrate or 200 g glycerol (depending on which C-source you prefer) and fill with water to 1 L. Autoclave and store at 4 °C.
3. Trace metal solution (PTM1) per L: 6.0 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.08 g NaI, 3.0 g $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.2 g $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.02 g H_3BO_3 , 0.5 g CoCl_2 , 20.0 g ZnCl_2 , 65.0 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g biotin, 5 mL H_2SO_4 . Weigh the chemicals in a beaker and fill with water to 1 L. Filter-sterilize through a 0.2 μm cutoff filter into a sterile flask and store at room temperature.
4. Base solution to set the pH: 2–3 M NH_4OH .

2.3 Methanol Solution for Pulses

For methanol pulses during batch cultivations use pure methanol supplemented with PTM1. For this purpose add 12 mL PTM1 solution into 1 L pure methanol, filter-sterilize this solution into a sterile bottle and store this solution at 4 °C.

2.4 Feeding Medium

Depending on the goal of the fed-batch cultivation (either biomass formation or induction of protein expression) different feeding media can be prepared. For *P. pastoris* glucose and glycerol are prominent C-sources for biomass formation, whereas methanol is used for the induction of protein expression.

1. Glucose feed per L: 275 g glucose monohydrate, 12 mL PTM1, 0.3 mL antifoam.
2. Glycerol feed per L: 250 g glycerol, 12 mL PTM1, 0.3 mL antifoam.
3. Methanol feed per L: 300 g methanol (use a balance), 4 mL PTM1, 0.3 mL antifoam.

The glucose and the glycerol feed can be sterilized via autoclavation; the methanol feed is sterile-filtered through a 0.2 µm cutoff filter into a sterile flask in order to avoid methanol evaporation.

2.5 Equipment

For a standard fed-batch experiment the following equipment is at least required:

1. Bioreactor (e.g., 5 L working volume glass bioreactor; Infors, Switzerland).
2. pH and pO₂ probe.
3. Air and oxygen lines.
4. Offgas analyzer (e.g., infrared cell for CO₂ and a zirconium dioxide sensor for O₂ concentration; DasGip, Germany).
5. Pumps and tubings for base and feed.
6. Balances (reactor balance, feed balance, base balance)— connected to the process information management system.
7. Process information management system (PIMS; e.g., Lucullus, SecureCell, Switzerland).
8. Spectrophotometer, centrifuge and dry oven for sample preparation.
9. HPLC for exact determination of methanol concentrations (e.g., Agilent Technologies, USA) equipped with a Supelco guard column, a SUPELCOGEL C-610H ion-exclusion column (Sigma-Aldrich, USA) and a refractive index detector (Agilent Technologies, USA).

3 Methods

3.1 Preculture of *Pichia pastoris*

Start a pre-culture of the *P. pastoris* strain of interest in 100 mL of YNB medium in 1 L baffled shaking flasks at 220 rpm and 28 °C for maximum 24 h (to guarantee good aeration only 1/10 of the total volume of the flask is filled with medium). The preculture is inoculated with 1 mL of frozen glycerol stock (*see* Notes 1-3).

3.2 Batch Cultivation in Bioreactors

After autoclaving the BSM in a bioreactor vessel, aseptically add the C-source (e.g., 40 g/L glucose or glycerol as final concentration in the vessel). Then adjust the temperature and the stirring speed to the desired values, before the pH in the bioreactor is adjusted to pH 5.0 using 25 % (v/v) ammonia solution (NH₄OH) by manually pumping the solution into the bioreactor. Aseptically transfer sterile PTM1 solution to the bioreactor (4.5 mL/L BSM; *see* Note 4). Aseptically transfer the preculture into a sterile inoculation flask (i.e., a vessel providing a connection to the bioreactor). The inoculum should be around 10 % of the final volume in the bioreactor.

Dissolved oxygen (dO₂) is measured with a sterilizable polarographic dissolved oxygen electrode. The pH is measured on-line with a sterilizable electrode and maintained constant with a step controller using 2–3 M NH₄OH which also represents the N-source during cultivation. The exact concentration of NH₄OH in the base bottle is determined by titration with 0.25 M potassium hydrogen phthalate (KHP; *see* Note 5). Base consumption is determined gravimetrically by putting the base bottle on a balance and recording the loss in weight over time. Set the cultivation temperature (e.g., 28 °C) and fix the agitation to the

¹When combining the sterile solutions for the preculture in a baffled shaking flask, work in the laminar flow hood and be careful to work sterile and avoid contaminations.

²If necessary, add antibiotics specific for the selection markers harbored by the strain (e.g., Zeocin, Kanamycin) to the preculture in appropriate concentrations to further reduce the risk of contamination.

³The glycerol stocks is prepared by mixing 1 mL of a fresh overnight culture of the respective *P. pastoris* strain with 0.5 mL sterile 75 % glycerol (v/v) and snap-freezing it in liquid N₂. The frozen glycerol stocks are then stored at –80 °C.

⁴Before inoculating the bioreactor with the appropriate amount of preculture, the following actions should be taken:

- Aseptically add the C-source to the sterile BSM in the bioreactor.
- Set the desired temperature (typically 28–30 °C) and stirring speed (e.g., 1,495 rpm).
- Set the pH value of the BSM to pH 5.0 with NH₄OH and note the amount of base which is required to determine the overall content in the bioreactor vessel.
- Add PTM1 aseptically to the cultivation broth.
- Calibrate the pO₂ electrode according to manufacturer's instructions.
- Adjust the weight of the bioreactor balance to the weight of the bioreactor content—the bioreactor weight is logged in the process information management system and by adjusting it correctly at this stage of the bioprocess the final data analysis will be facilitated.
- Note the “O₂ wet value,” which corresponds to the O₂ content measured in the offgas before inoculation. This value will be needed for the final data analysis.
- Aseptically inoculate the bioreactor with preculture (i.e., 100 mL for a final volume of 1 L cultivation broth).
- When taking samples, note the exact process time for the calculation of specific rates.

⁵We recommend taking at least two samples for the batch phase (right after inoculation and after the C-source is depleted) as well as at least two samples for each methanol pulse (before the pulse and after methanol depletion, which is indicated by a drop in the offgas signal). During the fed-batch phase we recommend taking samples every 4 h.

highest possible setpoint (e.g., 1,495 rpm) to guarantee good aeration. Aerate the culture with 2.0 vvm dried air (i.e., volume per volume per minute; in 1 L cultivation volume 2.0 vvm correspond to 2 L of dry air per minute). Measure the offgas of the culture by using an infrared cell for CO₂ and a paramagnetic cell for O₂ concentration. Temperature, pH, dO₂, agitation in the vessel, as well as CO₂ and O₂ in the offgas are measured on-line and logged in a process information management system.

3.3 Analysis of Growth-Parameters During Cultivations

Harvest 5 mL of culture broth by centrifugation in 10 mL glass tubes (4,300 × *g*, 4 °C, 10 min), wash the pellet twice with 5 mL deionized water and determine the dry cell weight (DCW) after drying at 105 °C to a constant weight in an oven (approximately 2–3 days; see Note 5). Optical density of the culture broth throughout the process is measured using a spectrophotometer at a wavelength of 600 nm (OD₆₀₀). Dry cell weight measurement and OD₆₀₀ have to be correlated to be able to use the measured OD₆₀₀ values for *q_s* adaptation in subsequent fed-batch cultivations (see Notes 6 and 7).

3.4 Batch Cultivation with Methanol Pulses

After the complete consumption of the C-source (e.g., glucose or glycerol), which is indicated by an increase of dissolved oxygen and a drop in offgas activity, perform the first methanol pulse of a final concentration in the bioreactor of 0.5 % (v/v). Following pulses are performed one after the other with 1 % (v/v) methanol as soon as methanol in the bioreactor is depleted (evident in the offgas analysis; Fig. 1).

To obtain the specific rates for substrate uptake during each pulse, a minimum of two samples has to be taken, one directly after methanol addition and the other before complete methanol depletion. The samples are used to determine the concentration of residual substrate, OD₆₀₀ and the dry cell weight. Determined values at the beginning and the end of the respective pulse are used to calculate an average value of the specific substrate uptake rate (*q_s*) according to Eq. 1 (in short: take a sample—pulse methanol—when the offgas signal drops, indicating depletion of methanol, take another sample—measure the exact methanol concentration in these two samples by HPLC—calculate the volumetric methanol uptake rate and relate it to the total biomass content at the latter sample point).

⁶For the base titration the following materials are required: base (KOH, NH₄OH), 0.25 M KHP, Bromothymol blue (indicator), burette and beaker with magnetic stirrer. Add 2 mL of base to the beaker (dilute NH₄OH 1:10) and use the burette to add 0.25 KHP. At the point of equivalence, the color of the indicator will turn from blue to grey and then to green. Calculate the molarity of the base according to:

$$\text{molarity\_base} = f \times \frac{\text{molarity\_KHP} \times \text{base\_consumption (KHP)}}{\text{volume\_base (diluted/before titration)}}$$

molarity_base (mol/L), molarity_KHP (mol/L), base_consumption (KHP) [mL], volume_base (mL), *f* = dilution factor of base before titration

⁷To be able to use the OD₆₀₀ values to set the feeding rate to the desired *q_s* setpoint, it is crucial to have a good and reliable calibration curve of the OD₆₀₀ and the biomass content (Dry Cell Weight) in (g/L). Before starting the fed-batch bioreactor cultivation, generate such a calibration curve by using the biomass from batch cultivations in different dilutions.

$$q_s \text{ (mmol/g/h)} = \frac{\frac{\Delta \text{MeOH(g)}}{\frac{\Delta \text{time (h)}}{\text{biomass (g)}}}}{M_{(\text{MeOH})} \times 1,000} \quad (1)$$

$M_{(\text{MeOH})} = 32.04$ (g/mol), biomass = amount of biomass in gram at the time point of the latter sampling

The parameters which can be determined by this strategy are: (1) adaptation time of *P. pastoris* the methanol, (2) the q_s during the adaptation pulse and (3) a maximum q_s for methanol ($q_{s \text{ max}}$) (see Note 8). We recommend performing at least four methanol pulses to obtain statistically significant parameters (see Note 9).

3.5 Fed-Batch

During fed-batch the dissolved oxygen (dO₂) signal is used to adjust air-in flow to keep levels >30 % dO₂ at all time points. In case air flow is not sufficient to keep this dO₂ level, pure oxygen is added. After the complete consumption of the substrate at the end of the batch phase (see Notes 10 and 11), which is indicated by an increase of dissolved oxygen and a drop in offgas activity, the feeding phase of the fed-batch feed is started. The feed rate is measured and controlled using a gravimetrically based PID flow controller (see Note 12). The culture is first adapted to methanol: the adaptation is performed at a q_s setpoint of 0.5 mmol/g/h (Fig. 2). As soon as the offgas signal (CO₂) gets constant, the q_s setpoint can be stepwise increased up to the predetermined $q_{s \text{ max}}$ of the respective strain (Fig. 2). To be able to analyze the physiology and the productivity of the *P. pastoris* strain at each q_s step, it is required to allow the cells to adapt to the respective q_s step before increasing it (see Notes 13 and 14).

⁸During cultivations, use the same photometer for OD₆₀₀ measurements as for the calibration curve. Do not switch photometers during the experiment.

⁹The strain characteristic parameters which can be analyzed by the batch experiment with methanol pulses are:

- time_{adapt}: time period from induction until the offgas (CO₂) has reached its maximum.
- $q_{s \text{ adapt}}$: specific uptake rate for methanol during the adaptation pulse.
- $q_{s \text{ max}}$: the maximum specific uptake rate for methanol during consecutive pulses.

¹⁰To get even more precise data for q_s , the methanol stripping from the bioreactor can be considered according to the Antoine's equation [12, 13].

¹¹For bioreactor cultivations, the C-source can be freely chosen depending on the research question (e.g., glucose, glycerol, sorbitol).

¹²In case a high cell density cultivation is envisioned, the BSM should be concentrated (e.g., twofold) to ensure the availability of enough salt throughout the cultivation process. Additionally, when performing a high cell density cultivation (e.g., more than 100 g/L DCW), the concentration of the C-source can be increased.

¹³The feeding profile based on a constant q_s value describes an exponential function. Of course the manual adjustment of the feeding rate to the desired q_s setpoint is laborious and cumbersome. However, this strategy does not require sophisticated equipment or soft sensor tools. Nevertheless, besides this manual adjustment, automatic feed forward feeding regimes are applicable. Similar to feeding strategies based on a predefined equation for a certain specific growth rate, also the specific substrate uptake rate can be controlled automatically assuming a constant yield coefficient on the substrate methanol. Based on the consumed methanol feed (with known concentration), which is determined gravimetrically via a feed balance, the amount of generated biomass is easily computable. To calculate the biomass yield coefficient on the substrate methanol for a certain strain, the here described methanol pulse strategy during batch cultivations can be used.

¹⁴For laboratories which are not equipped with an offgas analyzer, the methanol pulses can also be followed by the dissolved oxygen (dO₂) signal. After pulse addition, dO₂ declines and only rises again when methanol is depleted.

3.6 Substrate Concentrations

Samples are centrifuged ($20,000 \times g$, 15 min) and then concentrations of methanol are determined in cell free samples by HPLC. The mobile phase is 0.1 % H_3PO_4 with a constant flow rate of 0.5 mL/min and the system is run isocratic. Calibration is done by measuring standard points in the range of 0.1–10 g/L methanol.

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Fig. 1.

Experimental strategy for the fast determination of strain specific parameters of *P. pastoris* using a batch experiment with methanol pulses of 0.5 and 1 % (v/v). *continuous line*, carbon dioxide emission rate (CER); *circle*, calculated specific substrate uptake rate (q_s). Figure adapted with permission from [14]

Fig. 2. Fed-batch cultivation of a *P. pastoris* strains on methanol with a stepwise increase of q_s to $q_{s \max}$. *straight line*, set point for q_s ; *black dot*, calculated q_s values; *black triangle*, methanol concentration in the supernatant. The $q_{s \max}$ of the respective *P. pastoris* strain was determined with 1.94 mmol/g/h before. When this level is exceeded in fed-batch cultivations, methanol accumulates in the cultivation broth. Figure adapted with permission from [15]