SHORT PROTOCOL No. 38 I February 2020

A Guide to *Pichia pastoris* Fermentation in Single-Use Stirred-Tank Bioreactors

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Abstract

This short protocol describes the detailed procedure of *Pichia pastoris* fermentation in a BioBLU® 3f Single-Use Bioreactor controlled by a BioFlo® 320 bioprocess control system. We ran a fed-batch fermentation and started feeding upon the appearance of a significant dissolved oxygen spike, which indicated carbon source depletion. Finally, based on this fermentation strategy, a reasonably high-density *P. pastoris* culture was obtained in the single-use vessel. This short protocol can be very helpful for those who are new to *P. pastoris* fermentation in bioreactors.

Content

Fig. 1: BioFlo 320 bioreactor control system.

1. Introduction

Pichia pastoris is a methylotrophic yeast largely used for heterologous protein production in biotechnology. As an expression system, the main advantages of *P. pastoris* over *E. coli* are that the eukaryotic *P. pastoris* is capable of carrying out post-translational modifications, such as glycosylation, and meanwhile secreting the protein of interest. Compared to mammalian cell culture, *P. pastoris* displays much faster growth and requires a less expensive growth medium, making it ideal for cultivation in bioreactors. The objective of this short protocol is to provide a guide for running *P. pastoris* fermentation in bioreactors. Further optimization can be carried out based on the specific bioprocess needs.

2. Material and Methods

2.1 Yeast strain

The *Pichia pastoris* strain used in this study was constructed by LakePharma, Inc. It is a methanol-induced GFP secretion strain. However, since our focus was on yeast culture and biomass accumulation, we did not apply methanol induction or quantification of the GFP expression in this study.

2.2 Medium preparation

Three types of media were prepared for different applications in this study:

> One medium for the inoculum preparation in shake flasks (YM Broth);
> One medium for the early batch stage in the BioBLU 3f vessel (Tables 1 and 2), and
> One feeding medium for the fed-batch culture (Table 3).

2.2.1 Medium for inoculum preparation

Fully dissolve 21 g BD Difco™ Dehydrated Culture Medium: Yeast Mold Broth (YM Broth, BD 271120) in 1 L DI water, make sure the pH is within the range of 6.2 ± 0.2, and autoclave on liquid cycle for 20 min at 121 °C.

2.2.2 Media for batch fermentation

Basal salts medium (BSM) containing per liter (Table 1) [1]: PTM4 trace elements solution containing per liter (Table 2) [2]

Table 1: Basal salts medium (BSM) containing per liter excluding the carbon source.

Basal salts medium	
KH_2PO_4	42.9 g
$(NH_4)2SO_4$	5.17 g
K_2SO_4	14.33 g
$CaSO_4 \cdot 2\ H_2O$	0.6 g
$MgSO_4 \cdot 7\ H_2O$	5.71 g
H_2SO_4*	0.5 mL
PTM4 trace elements solution	4 mL

* Sulfuric acid (Certified ACS Plus, Fisher Scientific, Catalog No. A300-212, 95-98 w/w%)

Table 2: PTM4 trace elements solution containing per liter

PTM4 trace elements solution	
$CuSO_4 \cdot 5\ H_2O$	2 g
NaI	0.08 g
$MnSO_4 \cdot 4\ H_2O$	3 g
$Na_2MoO_4 \cdot 2\ H_2O$	0.2 g
Boric acid	0.02 g
$CaSO_4 \cdot 2\ H_20$	0.5 g
$CoCl_2$	0.5 g
$ZnCl_2$	7 g
$FeSO_4 \cdot 7\ H_2O$	22 g
Biotin	0.2 g
H_2SO_4	1 mL

2.2.3 The feeding medium

Table 3: Feeding medium containing per liter:

Feeding medium	
Glycerol	500 mL
PTM4 trace elements solution	4 mL

In this study, we prepared and transferred 1.5 L Basal salts medium to the BioBLU 3f vessel for the early batch stage. The Basal salts medium was prepared excluding the PTM4 trace elements solution. pH was adjusted to 5.0. After pH adjustment, per 1 L of the medium, 20 g glycerol (15.9 mL), and 0.3 mL of Antifoam 204 (Sigma-Aldrich, A6426) were added to the vessel before autoclave sterilization. We added the amount of antifoam corresponding to a 0.03 % (v/v) of the final volume, so for a BioBLU 3f with its 3 L working volume, 0.9 mL antifoam was needed. The PTM4 trace elements solution was sterilized through 0.2 µm membrane filters. The feeding medium was prepared excluding the PTM4 trace elements solution and sterilized in an autoclave. For both the Basal salts medium and the feeding medium, we added sterile PTM4 solution after the autoclaved liquid cooled down to room temperature in the biosafety cabinet.

2.3 Vessel setup and sterilization

The BioBLU 3f Single-Use Vessel has 4 x Pg 13.5 ports which can be used for installing DO sensors, pH sensors, stainless steel cooling fingers, etc. Besides, there is one harvest tube, one sample port, one thermowell, two liquid addition overlays, one liquid addition submerged, one gas sparge with filter, two exhausts with filters, one additional exhaust for pressure release during autoclaving, and four sealed baffles (Fig. 2).

We extended the liquid addition ports on the head plate with appropriate lengths of silicon tubing connected to a weldable C-Flex® tubing portion. In addition, to maintain the smooth flow of the liquid through the peristaltic pump, especially for base and feeding media with their high glycerol concentrations, a section of PharMed® tubing was added

between silicone tubing connections and applied to the peristaltic pumps on the BioFlo 320.

In this study, the liquid addition submerged port was used for inoculation. The two liquid addition overlay ports were used for separately adding the base and feeding media. We installed a pH sensor, a DO sensor, and a stainless steel cooling finger into three Pg 13.5 ports. After these installations and the addition of the tubing extensions, the medium filled BioBLU 3f fermenter was autoclaved for 20 min on the liquid cycle setting. Then we cooled it to room temperature and installed the exhaust condenser bracket onto the head plate. The detailed procedures for vessel setup can be found in the Eppendorf Application Note 408 [3].

2.4 Inoculum preparation

The clone of the *P. pastoris* strain was stored as glycerol stock in cryovials in the -80 °C freezer. Before inoculum preparation, we took one cryovial to thaw at room temperature for 2 hours. We prepared and sterilized 500 mL YM Broth (pH 6.2 ± 0.2) by autoclaving, and let it cool before allocating 150 mL of it to each of three 500 mL sterile Erlenmeyer flasks. Then we took two 500 µL suspension aliquots from the cryovial to inoculate two out of the three Erlenmeyer flasks, and put the three flasks into an Innova® S44i incubator shaker. We then set the temperature at 25°C and agitation at 200 rpm, and left them for 24 h for the inoculum to grow. The third Erlenmeyer flask containing only the fresh YM Broth was used as a sterility control. For inoculation, we transferred the entire 150 mL of growing culture from one Erlenmeyer flask into a pre-sterilized bottle, and pumped the *P. pastoris* suspension into the fermenter to reach an inoculation ratio of 10 % (v/v) in a starting volume of 1.5 L.

Fig. 2: The head plate of a BioBLU 3f Single-Use Fermenter.

2.5 Sensor calibration

We calibrated the pH sensor outside of the vessel, before sterilization, following the 2-point calibration method. We used a buffer at pH 7.00 to set ZERO, and a buffer at pH 4.00 to set SPAN.

We calibrated the DO sensor inside the vessel after sterilization, just before the inoculation. Before autoclave sterilization, when assembling the vessel, we filled the sensor with fresh electrolyte solution in its cap, which was separated from the medium by a permeable membrane at the tip. Six-hour sensor polarization was needed by connecting the sensor with the control station, which provided a polarizing voltage to establish an anode and a cathode within the sensor. pH and temperature of the fresh medium were adjusted to 5.0 and 25 °C, respectively, to emulate the actual *P. pastoris* culture condition. As for the pH calibration, DO calibration was performed by applying the 2-point calibration method. We sparged pure nitrogen at 3 SLPM (standard liters per minute) with the maximum 1,200 rpm agitation until the DO value stabilized, to set ZERO at 0 %, then we switched the gas supply from nitrogen to air at 3 SLPM with 1,200 rpm agitation until the DO value stabilized to set SPAN at 100 %.

2.6 Pump calibration

Pump calibration was performed before the run. The same tubing applied to the peristaltic pump head for liquid addition during fermentation should be used in pump calibration. Pump calibration was performed by pumping DI water into a fully filled piece of tubing for a set period of time, and tracking the water volume collected in the graduated cylinder at the end of tubing. Then the maximum pump speed specific to the tubing to be used was recorded in the system.

2.7 Process parameters

The process parameters used in this study are listed in Table 4.

Specifically, the DO cascade was set up as shown in Fig. 3. Agitation, air, and oxygen were all cascaded to DO. To meet the DO setpoint at 30 %, we increased the agitation from 300 to the maximum 1,200 rpm, corresponding to a DO output from 0 to 50 %. We set the air sparging rate at 0.8 SLPM to start with, and ramped it up to 3.0 SLPM which was 1 vvm (vessel volumes per minute), when the DO output increased from 50 to 65 %. At last, oxygen enrichment was applied to increase the flow rate of pure oxygen in the sparging gas stream from 0 to 3 SLPM, corresponding to the percentage of oxygen from the oxygen pipeline of 0 to 100 %.

Table 4: Process parameters used in *P. pastoris* fermentation in BioBLU 3f controlled by BioFlo 320 control station

Parameter	Configuration
Vessel	BioBLU 3f
Inoculation density	10 % (v/v)
Dissolved oxygen (DO)	30 %
Agitation	Magnetic drive, controlled by DO cascade, 1,200 rpm maximum
Gassing	Automatic gas flow and mix, controlled by DO cascade
Temperature	25 °C, cooling controlled by stainless steel cooling fingers
pH	5.0 ± 0.1, controlled by 30 % (v/v) ammonium hydroxide solution
Impeller	Three Rushton impellers
Sparger	Macrosparger
Feeding	Constant feeding rate at 0.4 mL/min during the fed-batch stage

Since acids were produced during fermentation, we only prepared 30 % (v/v) ammonium hydroxide solution as the base for pH control. For each run, 1 L base solution was prepared and sterilized through 0.2 µm membrane filtration. The pump used for base addition was specifically assigned as a base pump through the control station.

Feeding was manually initiated after we observed the DO spike indicating the depletion of glycerol, the carbon source, in the original medium in the bioreactor. We kept the feeding at a constant rate of 0.4 mL/min.

2.8 Optical density measurements

After DO sensor calibration and right before inoculation, 25 mL of fresh medium was taken from the vessel. One milliliter of medium was used to set blank for measurement of optical density at 600 nm on a Eppendorf BioSpectrometer® kinetic, and the rest was used to dilute the dense *P. pastoris* suspension collected in the later phase during fermentation. Samples were taken regularly during the fermentation.

3. Results

In the shaker, we saw active *P. pastoris* growth in the two Erlenmeyer flasks. The medium in the third flask which was set as a control stayed clear after 24 h.

We observed a significant DO spike at t = 21.5 h after inoculation in the bioreactor and started feeding at t = 22 h. With the appearance of DO spike indicating the depletion of the carbon source, agitation also showed a sharp drop (Fig. 4). Once feeding started, in order to maintain the DO at setpoint of 30 %, the agitation continuously climbed up until it reached the maximum of 1,200 rpm at t = 26 h. After that, the air sparging started to increase and reached 1.1 SLPM at t = 27.5 h, beyond which both agitation and air sparging decreased. No pure oxygen was called during the entire process. Base was added automatically when needed to maintain the pH at 5.0 (Fig. 5).

The *P. pastoris* growth curve is shown in Fig. 6. Obvious exponential growth took place after feeding started at t = 22 h.

The fermentation was stopped at t= 58 h with an OD_{600} of 287.3, because the final working volume of 3 L of the fermenter was reached. The growth curve shown in figure 6 indicates that the growth potential could have continued if feeding would have been continued.

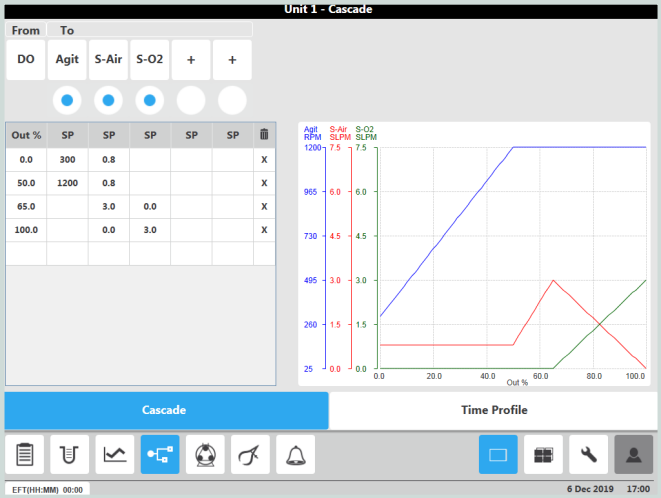

Fig. 3: DO cascade used in *P. pastoris* fermentation controlled by BioFlo 320.

Fig. 4: DO spike indicating carbon source depletion during the *P. pastoris* batch fermentation stage.

Fig. 5 The trends during the *P. pastoris* fed-batch stage.

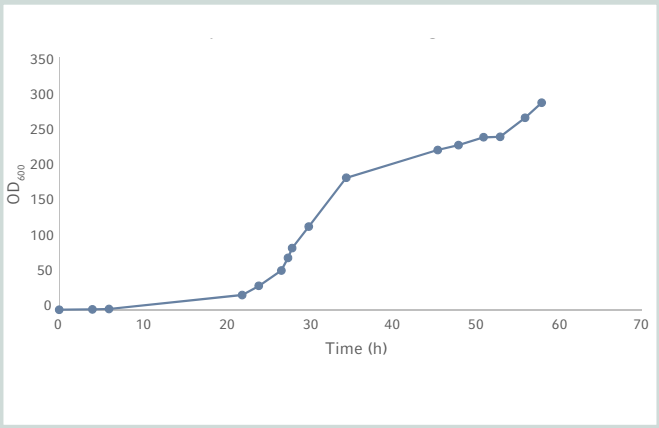

Fig. 6 Growth curve of *Pichia pastoris* in a BioBLU 3f single-use vessel under BioFlo 320 control station.

4. Literature

[1] Yang Z, and Zhang Z. Codon-optimized expression and characterization of a pH stable fungal xylanase in *Pichia pastoris*. Process Biochemistry (53), 80-87. 2017

[2] Edwards-Jones B, Aw R, Barton GR, Tredwell GD, Bundy JG, and Leak DJ. Translational arrest due to cytoplasmic redox stress delays adaptation to growth on methanol and heterologous protein expression in a typical fed-batch culture of *Pichia pastoris*. PLoS ONE (10), doi:10.1371/journal.pone.0119637. 2015

[3] Yang Y, and Sha M. A beginner's guide to bioprocess modes – batch, fed-batch, and continuous fermentation. Eppendorf Application Note 408. 2019

Ordering information

Description	Order no.
BioFlo® 320, all configured units include the same base control station	
Base control station	1379963011
BioBLU® 3f Single-Use Vessel, fermentation, macrosparger, 3 Rushton-type impellers, non-sterile, 1 piece	1386000900
Innova® S44i, incubated, 120 V, orbit diameter 2.5 cm (1 in)	S44I200005
Eppendorf BioSpectrometer® kinetic, 230 V/50-60 Hz	6136 000.002

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