eppendorf

APPLICATION NOTE No. 438

From Shaker to Fermenter: Methanol-free *Pichia pastoris* Protein Production Workflow

Ying Yang, Ma Sha
Bioprocess Applications Lab, Eppendorf, Inc., Enfield, CT, USA
Contact: bioprocess-experts@eppendorf.com

Abstract

The yeast *Pichia pastoris* has become an important and convenient workhorse for genetically engineered protein production in the biotechnology industry. Currently, most *P. pastoris* promoters used for efficient expression of heterologous proteins are derived from genes in the methanol metabolism pathway. For example, P_{AOX1}, the most widely used promotor, requires the switch to methanol feeding to activate protein production, referred to as methanol induction. Due to the flammable nature of methanol, this causes safety concerns in both academia and industry, especially when dealing with large volumes.

We explored the feasibility of a safer protein production alternative using a new *P. pastoris* strain carrying a strong methanol-independent promoter called pUPP. The pUPP promoter requires glycerol feeding for protein production, thus eliminating methanol induction and its affiliated safety risks. We carried out fed-batch *P. pastoris* fermentation runs for lipase production in the absence of methanol. This workflow was initiated in an Innova® S44i Biological Shaker for inoculum preparation, and followed with BioFlo® 120 and BioFlo 320 bioprocess control systems for bench scale fermentation.

We used 3 L BioBLU® 3f Single-Use Vessels as fermenters. At 27 h post inoculation, the BioFlo 120 controlled run reached a maximum OD_{600} of 183.8. The BioFlo 320 controlled run reached a final maximum OD_{600} of 229.8 at 46 h post inoculation. Maximum lipase activity was detected simultaneously with peak optical density, reflecting the positive correlation between yeast growth and protein production.

We conclude that this methanol-free *P. pastoris* fermentation workflow can be most advantageous for Eppendorf customers seeking a safer alternative to the traditional methanol induced *P. pastoris* protein production.

Introduction

The yeast *Pichia pastoris* has been developed as a highly competitive expression system for heterologous protein production. Compared to mammalian cell culture-based protein production systems such as CHO (Chinese Hamster Ovary) cells, the advantages of prokaryotic *P. pastoris* expression hosts include:

> Expedited growth, resulting in drastically shorter fermentation cycle,
> robustness,
> low cost of medium,
> absence of need for viral clearance,
> production of large quantities of the desired biomolecules at much higher concentrations [1, 2].

Currently most *P. pastoris* promoters used for efficient expression of heterologous proteins are derived from genes that code for enzymes in the methanol metabolism pathway. The most widely used promoter is P_{AOX1} [2, 3], which is strongly repressed in the presence of common carbon sources like glucose and glycerol. Upon depletion of the common carbon source, the promoter is de-repressed and capable of eliciting its full activity when induced by another carbon source, most typically methanol. However, due to its volatility and flammability, the addition of methanol to the bioprocess system brings significant safety concerns. Therefore, alternative promoters which do not require methanol induction to achieve high protein yield are much in demand.

Here we used a *P. pastoris* strain carrying a strong and constitutive promoter pUPP which can rely on glycerol as the only carbon source, meaning that both biomass growth and protein expression are supported by glycerol consumption, whereas no methanol is needed. The objectives of this study are: (1) to carry out fed-batch *P. pastoris* fermentation with the novel methanol-free induction technology for protein production, here in this case, lipase expression and secretion; and (2) to demonstrate the feasibility of using the Innova S44i Biological Shaker, the two benchtop bioprocess control systems BioFlo 120 and BioFlo 320, and the BioBLU 3f Single-Use Vessel, for high-density *P. pastoris* fermentation and protein production workflow.

Material and Methods

Yeast strain

The yeast strain *Pichia pastoris* Bg10-pJAG-Lip1 used in this study was constructed by BioGrammatics, Inc. This strain contains a lipase expression construct and a constitutive pUPP promoter (also known as P_{GCW14}) which is free of methanol regulation in the production of heterologous protein [4]. Upon receipt, we streaked the *P. pastoris* cells on a YPD agar plate, incubated at 28 °C for 48 h, and picked a healthy single colony to inoculate a 250 mL shake flask containing 75 mL fresh YPD medium. We transferred the shake flask into the Innova S44i Biological Shaker at 28 °C with 200 rpm agitation. After 48 h, we prepared the glycerol stock in multiple cryogenic vials by adding 600 µL of the actively growing *P. pastoris* suspension from the shake flask to each of the cryogenic vial containing 400 µL 75 % (v/v) sterile glycerol. Cells were well mixed before being transferred into the freezer at -80 °C (New Brunswick Innova U360, Eppendorf) for future use.

Media preparation

We used two types of growth media in this study, YPD (Yeast extract-Peptone-Dextrose) and BMGY (Buffered Glycerol-complex Medium).

For YPD medium, both agar and liquid media were prepared. The agar YPD medium was made by dissolving 10 g yeast extract (Fisher BioReagents™, BP1422-500), 20 g peptone (BD Gibco™ Bacto™ Proteose Peptone No. 3, 211693), 20 g glucose (Sigma-Aldrich®, G7021), and 15 g agar (BD Difco™ Plate Count Agar, 247940) per 1 L DI water through boiling, and autoclaved at 121 °C. We poured agar plates to streak the *P. pastoris* cells after receipt as described above. The liquid YPD medium was prepared by dissolving all the compounds except agar at the same amount per 1 L DI water and autoclaved at 121 °C. The liquid medium was used when preparing the glycerol stock.

BMGY broth was prepared for bioreactor fermentation. One litre of BMGY broth was made as follows:

> 10 g yeast extract, 20 g peptone and 7.5 mL glycerol (Fisher BioReagents™, BP229-1) were disslved in 800 mL DI water and autoclaving at 121 °C.
> Next we prepared 100 mL 1 M potassium phosphate buffer (pH 6.0) by dissolving 2.405 g K_2HPO_4 (Fisher Chemical™, P228-500) and 11.73 g KH_2PO_4 (Sigma Aldrich, P5655) in 100 mL DI water, and sterilized the broth by passing it through a 0.2 µm membrane.

We also prepared 10x YNB (Yeast Nitrogen Base, with ammonium sulfate) solution by dissolving 13.4 g of the YNB powder (Invitrogen™, Q300009) in 100 mL DI water and sterilizing through 0.2 µm membrane filtration into the cooled broth to make a total volume of 1 L.

The fed-batch fermentation feeding medium was a 50 % (v/v) glycerol solution, sterilized by autoclaving in the feeding bottle and aseptically connected to the liquid addition port on the head plate of the bioreactor before the run.

Inoculum preparation

For each procedure, we removed one *P. pastoris* cryogenic vial from the -80 °C freezer and thawed it under ambient temperature. We prepared two 500 mL Erlenmeyer flasks, each containing 150 mL fresh BMGY medium, and inoculated 500 µL of the *P. pastoris* glycerol stock from the vial into each of the two flasks. A third control Erlenmeyer flask contained 150 mL fresh BMGY medium without inoculation. We transferred the three flasks into the Innova S44i Biological Shaker for the inoculum to grow at 28 °C with 200 rpm agitation. After 48 h, the broth in the two Erlenmeyer flasks which had been inoculated turned turbid, indicating active growth of *P. pastoris*. The medium in the control flask should stay clear, demonstrating sterility of the medium and aseptic manipulation.

After 48 h shaking culture, we picked one of the two flasks containing 150 mL actively growing *P. pastoris* suspension and measured its optical density at 600 nm with the fresh BMGY medium as blank using an Eppendorf BioSpectrometer®. The OD_{600} of this shake flask culture was 30.9. We then carefully poured the 150 mL suspension into two 500 mL sterile bottles. The 75 mL inoculum was pumped at maximum speed into each vessel. The schematic flowchart for glycerol stock preparation and bioreactor fermentation is illustrated in Fig. 1.

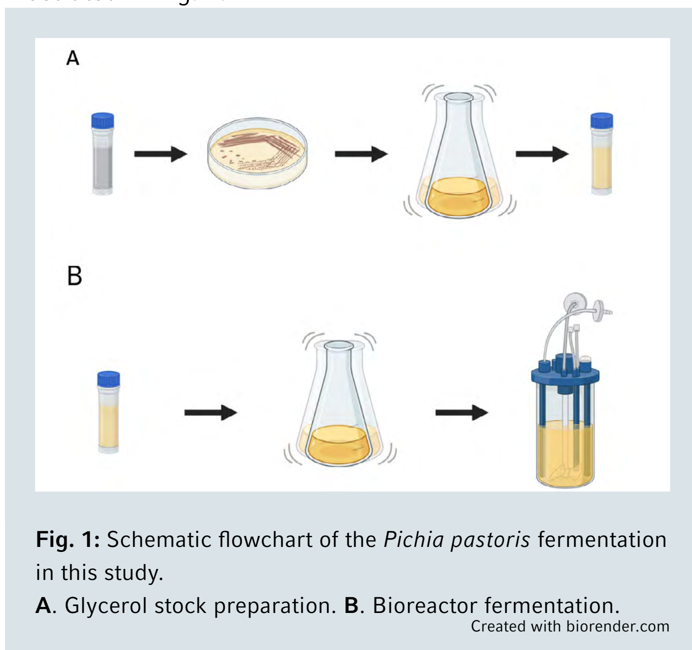

Fig. 1: Schematic flowchart of the *Pichia pastoris* fermentation in this study.
A. Glycerol stock preparation. **B**. Bioreactor fermentation.
Created with biorender.com

BioBLU® 3f Single-Use Bioreactor and vessel setup

In this study, we performed all fed-batch fermentations in BioBLU 3f Single-Use Bioreactors. The vessel has rigid walls with a working volume range of 1.25 – 3.75 L and allows a maximum agitation of 1,200 rpm. It is equipped with a macrosparger and three Rushton-type impellers, specifically designed for robust microbial applications.

All fed-batch fermentations started with an early-stage batch run at 1.5 L working volume in BMGY broth. Towards the end of the fed-batch, the final working volume was around 3 L. We added 0.9 mL Antifoam 204 (Sigma-Aldrich, USA) to the BMGY broth when filling the BioBLU 3f vessel before autoclaving to reach a final antifoam concentration of 0.03 % (v/v).

We used a pH/Redox ISM® sensor for pH monitoring and an analog polarographic dissolved oxygen (DO) sensor for DO measurements (Mettler Toledo®, Switzerland). Both sensors are 12 mm in diameter with 225 mm insertion depth. The two sensors were installed on the head plate of the BioBLU 3f vessel through the two Pg 13.5 ports. A stainless-steel cooling finger was installed through a compression fitting taking another Pg 13.5 port. We extended three liquid addition ports appropriately for connection with the external bottles.

Fig. 2 illustrates the BioBLU 3f Single-Use Vessel as it appears before autoclave sterilization. We manually applied the Ni-plated open jaw Hoffman tubing clamp (Eppendorf order No. P0160-4830) to each of the head plate tubing which connects to a submerged port into the medium. These ports include gas sparger, submerged liquid addition line, harvest port, and sampling port. We tightened these metal clamps to prevent the medium from refluxing into the tubing, filters, and possibly ejecting during high-temperature high-pressure autoclaving. Additionally, to ensure effective venting during autoclaving, the overlay gas port on the BioBLU 3f head plate should be left open, as marked by a "Do not clamp" sign pre-attached to this specific port.

We autoclaved the medium-filled vessel and let it cool to 28 °C. We then added sterile 1 M potassium phosphate buffer and 10x YNB solution to the vessel to complete the BMGY broth preparation as described earlier. Upon completion of DO sensor calibration, the inoculation bottle, the feeding bottle, and the base bottle were aseptically connected before inoculation to the liquid addition ports on the vessel head plate through a SCD®-II Sterile Tubing Welder (Terumo BCT, USA). The detailed setup of the vessel can be found in a previously published application note [5].

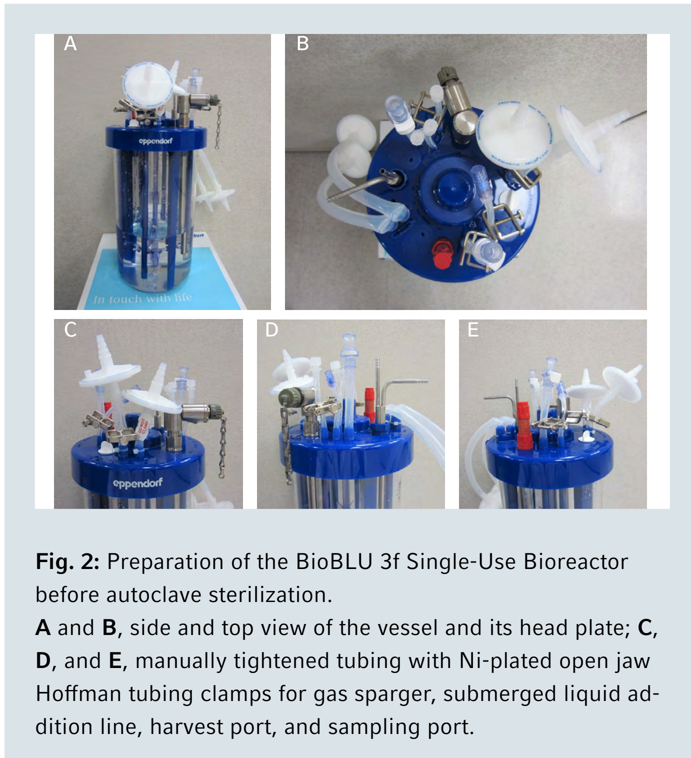

Fig. 2: Preparation of the BioBLU 3f Single-Use Bioreactor before autoclave sterilization.
A and **B**, side and top view of the vessel and its head plate; **C**, **D**, and **E**, manually tightened tubing with Ni-plated open jaw Hoffman tubing clamps for gas sparger, submerged liquid addition line, harvest port, and sampling port.

Sensor calibration

We followed the same sensor calibration protocols for both BioFlo 120 and BioFlo 320 bioreactors, calibrating the pH sensor outside of the vessel before sterilization. We used the 2-point calibration method by setting ZERO using buffer at pH = 7 and setting SPAN using buffer at pH = 4.

We calibrated the pre-polarized DO sensor after autoclaving with the sterile BMGY broth in the vessel. We recommend calibration of the DO sensor under the same condition as during the actual fermentation procedure. Therefore, we checked to make sure the pH was at 6.0, set the agitation at maximum 1,200 rpm, and set the temperature at 28 °C. A 2-point calibration method was also applied. We sparged pure nitrogen at 1 VVM, here in this case 1.5 SLPM, until the DO value stabilized to set ZERO at 0 %; then switched the gas supply to air under the same flow rate, waited till the DO value stabilized again, to set SPAN at 100 %.

Pump calibration

Prior to the run, the pumps were calibrated on both control systems. The same tubing applied to the peristaltic pump head for liquid addition during fermentation should be used for the pump calibration. It is recommended to add a section of PharMed® tubing (Saint-Gobain®, France) between silicone tubing connections and fit it to the peristaltic pumps for better chemical compatibility especially for base addition. Pump calibration was performed by pumping DI water into a fully filled section of tubing for a set period of time (3 min for both control systems) and tracking the water volume collected in a graduated cylinder at the end of tubing. Then the maximum pump speed specific to the tubing used can be recorded in the system. Pump calibration is critical here for the addition of feeding medium and base during fermentation.

Process parameter setup from shaker to fermenters

The bench scale *P. pastoris* fermentations were maintained at 28 °C, pH 6.0, and 30 % DO. Most of the process parameters retained for the two bioprocess control systems (see Table 1). To show the entire workflow, Table 1 also includes the parameter setup of the early inoculum preparation. The key equipment used in this workflow is illustrated in Fig. 3.

For the BioFlo 120, heating was controlled by an external heat blanket. The DO cascade was designed to maintain the DO at 30 % by first accelerating the agitation from 300 to 1,200 rpm, then increasing the air sparging rate from 0.8 to

Table 1. Key process parameters applied to *Pichia pastoris* fermentation and protein production workflow.

Parameter	Configuration
Inoculum preparation	
Shaker	Innova S44i Biological Shaker
Temperature	28 °C
Agitation	200 rpm
Flask	500 mL Erlenmeyer flasks
Volume	150 mL in each Erlenmeyer flask
Duration	48 h
Bench scale fermentation	
Controller	BioFlo 120 and BioFlo 320
Vessel	BioBLU 3f
Inoculation density	5 % (v/v), 75 mL inoculum to an initial 1.5 L working volume
Dissolved oxygen (DO)	30 %, maintained by DO cascade
Agitation	Magnetic drive, maximum 1,200 rpm, controlled by DO cascade
Gassing	Automatic gas flow and mix, controlled by DO cascade
Temperature	28 °C, cooling controlled by a single stainless-steel cooling finger
pH	6.0 ± 0.1, controlled by the addition of 30 % (v/v) sterile ammonium hydroxide solution
Impeller	Three Rushton impellers
Sparger	Macrosparger
Feeding	Manually triggered by the DO spike, then at a constant feeding rate of 0.4 mL/min of 50 % (v/v) glycerol solution for the rest of fed-batch fermentation

3.0 SLPM, and finally enriching oxygen in the sparged gas stream from 0 to 100 %. These three steps corresponded to the DO output of 0-50 %, 50-65 %, and 65-100 %, respectively, which are fixed in the BioFlo 120. Based on our preliminary studies, to better support yeast growth, we found the importance of keeping a minimum air sparging rate at 0.8 SLPM throughout the run rather than starting from zero air flow. The detailed DO cascade setup in the BioFlo 120 is shown in Fig. 4.

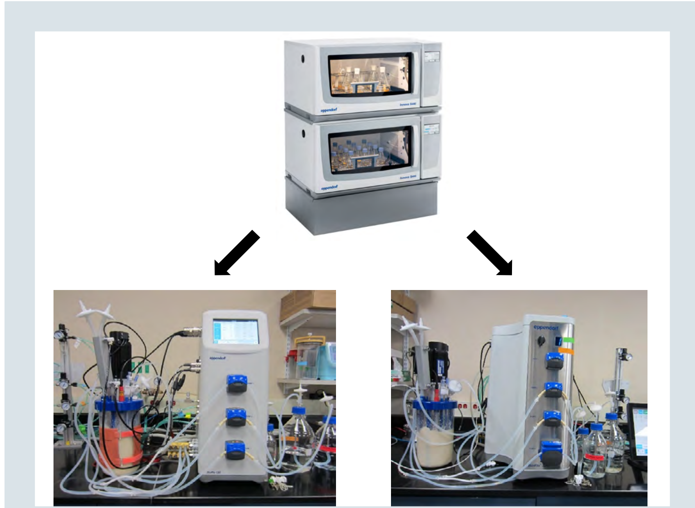

Fig. 3: Key equipment used in this *Pichia pastoris* fermentation and protein production workflow.
Top: Innova S44i Biological Shaker; **bottom left:** BioFlo 120 bioprocess control station; **bottom right:** BioFlo 320 bioprocess control station).

For the BioFlo 320, both cooling and heating were controlled by running water through the stainless-steel cooling finger. We set the DO output between 0 % and 100 %, and built a customized DO cascade the same as it was for BioFlo 120 (Fig. 5).

For a more direct comparison, Table 2 lists some configuration differences between BioFlo 120 and BioFlo 320 in this *P. pastoris* fermentation. Besides the aforementioned heating and DO cascades, the most distinctive differences are the gas sparging and pumps. For BioFlo 120, the sparge gas module has 1 TMFC (thermal mass flow controller) which has 4 solenoid valves but only allows one gas to flow through at a given time point without pre-mixing. Therefore, towards the later stage of aerobic *P. pastoris* fermentation when oxygen enrichment takes place, both air and oxygen are called, but only one gas is sparged to the bioreactor at a given time

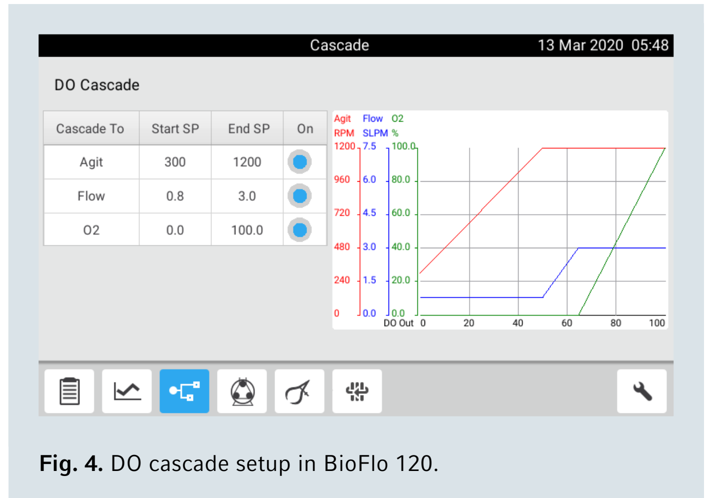

Fig. 4. DO cascade setup in BioFlo 120.

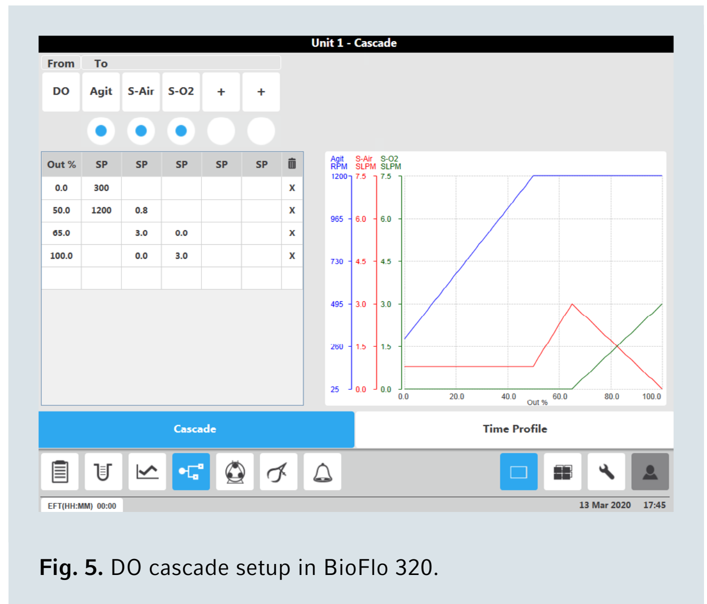

Fig. 5. DO cascade setup in BioFlo 320.

point depending on the real-time DO output. The BioFlo 320 is equipped with 4 TMFCs. Different kind of gases, air and oxygen in this case, can be called simultaneously at different flow rates and mixed together, thus allowing a more comprehensive and precise control of gas sparging during the process. Furthermore, compared to the three fixed speed pumps on BioFlo 120, BioFlo 320 has three variable speed pumps and a fourth one with much more powerful performance, greatly contributing to the flexibility and efficiency of the bioprocess.

An obvious DO spike indicated the depletion of the carbon source, glycerol in this case, in the batch stage of *P. pastoris* fermentation. When glycerol depletion occurred, the

Table 2. Differences in bioprocess control between BioFlo 120 and BioFlo 320 in this study.

Parameter	BioFlo 120	BioFlo 320
Heating	An external heating blanket	Heated water circulating through the stainless-steel cooling finger
Gas sparging	1 TMFC (0.04-20 SLPM)	4 TMFCs (0.04-20 SLPM)
DO cascade	Fixed DO output range for a series of defined parameters: agitation, air, and oxygen	Can be completely customized, the DO output ranges, and the associated parameters are all self-defined
Pumps	Three front-mounted pumps (type 114DV) which run at fixed speed with 0-100 % duty cycle	Four pumps which can run at variable speeds, the top three are type 114DV pumps, and the fourth has a larger capacity (type 314D) and runs up to 4x faster
Inoculation	Through one of the three same pumps	Through the fourth pump which leads to a very fast inoculation process

metabolic rate of *P. pastoris* cells significantly slowed down including the consumption of oxygen. As a result, a DO spike took place. Here the DO spike triggered control was achieved by manual observation and handling with experience from previous runs based on EFT (elapsed fermentation time). We manually turned on the feeding pump at a constant pump rate of 0.4 mL/min right after we detected the DO spike on the trend page collected by both control systems.

Biomass formation – optical density measurement

Upon completion of DO calibration and right before inoculation, we took a sample of 30 mL fresh BMGY broth from one vessel, and used 1 mL of this medium to set the blank for optical density measurement at 600 nm using an Eppendorf BioSpectrometer. The remaining volume was saved as the diluent for the yeast suspension during the run. We took suspension sample at 11 time points, 0, 3, 6, 21, 24, 27, 29, 46, 48, 51, and 54 h after inoculation, for optical density measurement.

Protein production – lipase activity assay

The Lipase Activity Assay Kit (colorimetric) used in this study was purchased from Abcam® (ab102524). Since lipase was secreted from the *P. pastoris* cells, we took one extra mL suspension at the last 7 sampling time points for OD measurements, pelleted the yeast cells down by centrifugation in a MiniSpin® plus microcentrifuge at 14,000 rpm for 90 s, and collected the supernatants for lipase activity colorimetric assay. The basis of this assay is lipase hydrolyzation of a triglyceride substrate to form glycerol which can be quantified enzymatically by monitoring a linked colorimetric change in the OxiRed probe absorbance at 570 nm. We followed the assay protocol to first load a series of standards, samples, background control samples, and positive controls into a 96-well plate, then adding the reaction mix and background reaction mix respectively to the designated wells. The OD_{570} of the plate was measured by Epoch™ Microplate Spectrophotometer (BioTek Instruments) in kinetic mode for 60 min. The standard curve was drawn and the lipase activity of each supernatant sample was calculated accordingly. The detailed protocol is available when purchasing the assay kit or accessed on the Abcam website [6].

Results

We ran the *Pichia pastoris* fed-batch fermentation two times at 28 °C, pH 6.0, and a DO level of 30 % in BioBLU 3f bioreactors, one controlled by a BioFlo 120, and the other by a BioFlo 320 bioprocess controller. We maintained DO by applying a customized DO cascade and initiated feeding right after observing the DO spike. Throughout the fermentation, we took intermittent samples for optical density measurements and lipase activity assays to evaluate the yeast growth and secreted protein production.

Bioprocess trends in the BioFlo 120 and BioFlo 320 bioprocess control systems

As described previously, a DO spike during fermentation indicates the depletion of the carbon source in the broth and is the signal for initiating feeding. We observed a significant DO spike at 9.25 h in both runs, indicating a synchronized growth and metabolism pattern of *P. pastoris* in the two vessels. Relative to a DO set point at 30 %, the peak of the DO spike was 35 % and 50 % in BioFlo 120 and the BioFlo 320, respectively. Right before the appearance of DO spike, agitation was ca. 800 rpm and still ramping up. The DO spike was accompanied by a sharp drop of agitation, indicating the largely reduced demand of oxygen since the metabolism of *P. pastoris* slowed down. Immediately after feeding was initiated, yeast growth quickly resumed, which caused the DO concentration to decrease first and soon recover with agitation continuing its upward trend.

For both control systems, the agitation reached its maximum at 1,200 rpm at t = 11.5 h and maintained at this speed for the rest of the fermentation. The air sparging rate ramped

up from 0.8 to 3.0 SLPM in the next 3 hours before oxygen enrichment took place at t = 14.5 h. From then on, the trends collected in BioFlo 120 and 320 started to show some difference. In the BioFlo 120 controlled fermenter, oxygen enrichment gradually increased from 0 to 10 % in the sparging gas for the next 7 hours till t = 21.5 h and then started to decline back to 0 % at t = 36 h. Therefore, oxygen enrichment lasted for a total of 21.5 h. After that, the air flow rate started to decrease from 3 to 0.9 SLPM from t = 36 h towards the end of the run at t = 54 h. In the BioFlo 320 controlled run however, oxygen input was only called intermittently at a flow rate of 0.1 SLPM corresponding to 3.3 % in the 3 SLPM sparging gas stream for 1.5 h until t = 16 h, then no oxygen was needed for the rest of the run. The air flow rate started to drop from 3 to 1.6 SLPM during the next 2.5 h till t = 18.5 h and then slowly recovered to 2.4 SLPM at t = 29 h. Beyond that, the air flow rate declined again and was at 1.8 SLPM when we ended the fermentation at t = 54 h.

Yeast growth and lipase activity

Based on the optical density measurements, growth curves are drawn for both fermentations (Fig. 6). According to the growth curve, *P. pastoris* grew faster during the exponential growth phase under BioFlo 120 with maximum OD_{600} of 183.8 at t = 27 h. After that, the stationary phase and death phase were observed in the BioFlo 120 culture and OD_{600} started to decline till the end of fermentation. However, the growth pattern of the BioFlo 320 culture was different. Although its specific growth rate was lower than the BioFlo 120 counterpart during the early exponential growth, yeast biomass continued to accumulate till t = 46 h with the maximum OD_{600} at 229.8 in the BioFlo 320 culture before entering the stationary phase.

The growth curve correlates well with the culture's real-time oxygen demand as described earlier. In the first half of the fermentation, after air flow reached its maximum of 3 SLPM at t = 14.5 h, up to 10 % oxygen enrichment was observed for the BioFlo 120 culture and the enrichment lasted for 21.5 h. The early high demand of oxygen supported robust exponential *P. pastoris* growth controlled by BioFlo 120. However, for BioFlo 320 culture, oxygen enrichment was minimal, but air sparging rate maintained at a relatively high level despite some fluctuations throughout the entire fermentation, which resulted in a long and steady growth of *P. pastoris* till t = 46 h.

For lipase activity, we drew developed a linear standard curve based on the assay protocol to correlate the concentration of glycerol loaded in each well with the OD_{570} reading associated with the OxiRed probe at the end of 60 min incubation [6]. The equation of this linear standard curve is OD_{570} = 0.1514 x glycerol concentration (nmol) with a R-square of 0.9937. Therefore, the lipase activity we can be calculated the lipase activity accordingly from the colorimetric readings collected from each well, indicating the glycerol released from triglyceride hydrolysis.

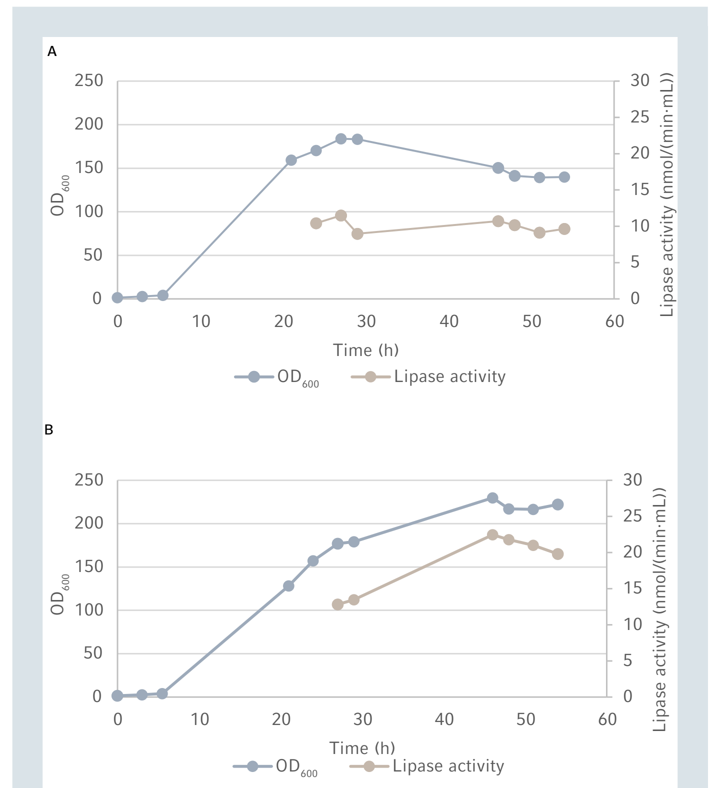

Fig. 6. Growth curves of *Pichia pastoris* and the activities of the secreted lipase in BioBLU 3f Single-Use Vessel controlled by BioFlo 120 (A) and BioFlo 320 (B) bioprocess control systems.

We found the maximum lipase activity was 11.5 nmol/(min·mL) at t = 27 h for BioFlo 120 and 22.5 nmol/(min·mL) at t = 46 h for BioFlo 320. We notice that for both control systems, maximum lipase activity was detected simultaneously with the peak of *P. pastoris* optical density in each vessel.

Since the pUPP is a constitutive promoter, heterologous protein expression and secretion take place at the same time with *P. pastoris* growth. Therefore, it is not surprising to see

that lipase activity and yeast biomass are generally positively correlated, which provides us an easier approach to high protein yield through simply boosting yeast growth. This is very different from growing *P. pastoris* which has the widely used adopted methanol-induced promoter like such as the alcohol oxidase I promoter P_{AOX1} for protein production. As a host with such a methanol regulated promoter, *P. pastoris* needs to go through separate stages for biomass accumulation first and then heterologous protein production by methanol induction, a strategy which poses more challenges in experimental design.

Conclusion

This study successfully demonstrates a workflow of methanol-free induction for heterologous protein production in *P. pastoris* from shake flask culture to bioreactor fermentation. The Innova S44i Biological Shaker, the BioFlo 120 and the BioFlo 320 control systems, together with the BioBLU 3f Single-Use Vessels, are all capable of supporting such an innovative and convenient fermentation bioprocess.

With a strong and constitutive promoter independent of methanol regulation, the experimental design for such fed-batch *P. pastoris* fermentation is safer, simpler, and shorter than the traditional P_{AOX1} induced protein production. *P. pastoris* reached maximum OD_{600} of 183.8 at t = 27 h under BioFlo 120, and maximum OD_{600} at 229.8 at t = 46 h under BioFlo 320. Maximum lipase activity was detected simultaneously with peak optical density, indicating a positive correlation between yeast growth and protein production.

This detailed example of methanol-free induction in *P. pastoris* fermentation can serve as a useful reference for customers seeking a safer alternative to the traditional methanol induced bioprocess. We assert that the methanol-free *P. pastoris* strain with robust and safe expression system can grow into a key competitor to traditional protein production technologies dominated by the use of methanol induced strains.

Literature

[1] *Pichia pastoris* Revisited. Genetic Engineering and Biotechnology News, Vol 34(11). 2014

[2] Ahmad M, Hirz M, Pichler H, Schwab H, Protein expression in *Pichia pastoris:* recent achievements and perspectives for heterologous protein production. Applied Microbiology and Biotechnology 98(12), 5301-5317. 2014

[3] Gonçalves AM, Pedro AQ, Maia C, Sousa F, Queiroz JA, Passarinha LA. *Pichia pastoris*: A recombinant microfactory for antibodies and human membrane proteins. Journal of Microbiology and Biotechnology 23(5), 587-601. 2013

[4] Liang S, Zou C, Lin Y, Zhang X, Ye Y. Identification and characterization of P_{GCW14}: a novel strong constitutive promoter of *Pichia pastoris*. Biotechnology Letters (35), 1865-1871. 2013

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

[6] ab102524 Lipase Activity Assay Kit (Colorimetric) protocol booklet.
Available online at https://www.abcam.com/lipase-assay-kit-colorimetric-ab102524.html

eppendorf

Ordering information

Description	Order no.
Innova® S44i, incubated, 120 V, orbit diameter 2.5 cm (1 in)	S44I200005
Innova® U360, 360 L, ULT freezer, 115 V/60 Hz	U9425-0000
New Brunswick™ Excella® E24, orbit diameter 1.9 cm (3/4 in), 120 V/60 Hz	M1352-0000
Eppendorf BioSpectrometer® kinetic, 230 V/50-60 Hz	6136 000.002
BioFlo® 320, all configured units include the same base control station	
Base control station	1379963011
BioFlo® 120	
Advanced control station bundle, TMFC 20 SLPM	B120ACS000
BioBLU® 3f Single-Use Bioreactor, fermentation, macrosparger, 3 Rushton-type impellers, autoclavable	1386000900
Bioprocess system Accessories	
Agitation motor for BioBLU® 3f	1386080000
Water manifold kit	M1386-9909
Cooling finger for BioBLU® 3f, stainless steel	M1386-0555
Exhaust condenser kit	M1386-9905
Exhaust condenser bracket, BioBLU® 3f	1386930400
Heat blanket for BioBLU® 3c/5c/5p/3f	M1379-8116
Heat blanket adaptor for BioBLU® (to be used with BioFlo® 120)	1386811900
pH/Redox sensor, Mettler Toledo®, InPro 3253i, ISM®, 225 mm	P0720-6657
DO sensor, Mettler Toledo®, InPro 6830, angled T-82 connector, 220 mm	P0720-6282
Ni-plated open jaw Hoffman tubing clamp	P0160-4830
Centrifuge MiniSpin® plus, non-refrigerated, with Rotor F-45-12-11	5453 000 015

Your local distributor: www.eppendorf.com/contact
Eppendorf AG · Barkhausenweg 1 · 22339 · Hamburg · Germany
eppendorf@eppendorf.com · www.eppendorf.com

www.eppendorf.com/bioprocess

Abcam® is a registered trademark of Abcam PLC, UK. Sigma-Aldrich® is a registered trademark of Sigma-Aldrich Co. LLC, USA. ISM® and Mettler-Toledo® are registered trademarks of Mettler-Toledo GmbH, Switzerland. SCD® is a registered trademar of Terumo BCT, Inc, USA. PharMed® and Saint-Gobain® are registered trademarks of Saint-Gobain Performance Plastics Inc., USA. Difco™ Laboratories Incorporated , USA. Invitrogen™ is a trademark of Life Technologies Corporation, USA. Gibco™ and Bacto™ are trademarks of Beckton, Dickinson and Company, USA. BioReagents™ and Fisher Chemical™ are trademarks of Fisher Scientific Company L.L.C., USA. Epoch™ is a trademark of BioTek Instruments Inc., USA. Eppendorf®, the Eppendorf Brand Design, BioBLU®, MiniSpin®, and Eppendorf BioSpectrometer® are registered trademarks of Eppendorf AG, Germany. BioFlo®, Excella®, and Innova® are registered trademarks of Eppendorf, Inc., USA. New Brunswick™ is a trademark of Eppendorf AG, Germany.
All rights reserved, including graphics and images. Copyright © 2021 by Eppendorf AG.