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Human IgG Fc Production Through Methanol-Free *Pichia pastoris* Fermentation

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ABSTRACT

Nowadays, therapeutic monoclonal antibodies (mAbs) are predominantly produced with mammalian cell culture systems such as those using Chinese hamster ovary (CHO) cells. Efforts are underway to reduce the costs of this process to meet the increasing global demand in biopharmaceuticals; meanwhile, cheaper and faster expression systems are being investigated as alternatives. The yeast, *Pichia pastoris,* has become a substantial workhorse for recombinant protein production. However, the N-linked glycosylation in *P. pastoris*, namely high mannose glycosylation, is significantly different from that in CHO or other mammalian cells, including human cells. In this study, a SuperMan5 strain of *P. pastoris* was constructed using Pichia GlycoSwitch® technology to successfully produce a more mammalian-like immunoglobulin G (IgG) fragment crystallizable (Fc), which showcases the potential of *P. pastoris* as a next-generation mAb production platform. Importantly, in this study, a strong methanol-independent promoter, P_{UPP}, was applied, which only requires glycerol feeding for protein production. Most *P. pastoris* promoters used for protein expression are derived from genes in the methanol metabolism pathway, creating safety concerns due to the flammable nature of methanol, especially at large scale. Here, a fed-batch SuperMan5 *P. pastoris* fermentation was carried out in which methanol induction, as well as its affiliated safety risks, were eliminated. Overall, this study provides insights into the development of safe and cost-effective industrial mAb production approaches independent of mammalian cell culture.

INTRODUCTION

The yeast, *Pichia pastoris*, is one of the most robust and cost-effective eukaryotic expression systems for heterologous protein production. Compared to prokaryotic platforms such as *Escherichia coli*, the *P. pastoris* eukaryote can carry out more complicated post-translational modifications including folding, disulfide formation, and glycosylation.[1,2] When compared to mammalian cell culture-based protein production systems such as those using CHO cells, the advantages of expression with *P. pastoris* include: much faster growth resulting in drastically shorter production cycles, robustness of cultures, cheaper media, easier genetic manipulation to create and improve production strains, reduced virus concerns, and significantly lower overall production cost.[3-5]

Glycosylation is a common post-translational modification of many proteins during which carbohydrate moieties are covalently linked to specific amino acid residues in the protein molecule. Approximately 60% of human proteins are glycosylated, and the great diversity of glycans significantly contributes to immunogenicity and many physiological functions of the human body.[6,7] Although much of the glycosylation pathway is conserved from yeast to humans, *P. pastoris* mainly undergoes high/hyper-mannose-type N-glycosylation, meaning its N-linked glycans are largely composed of a single type of sugar monomer, mannose, which prevents *P. pastoris* from being a qualified expression system for therapeutic mammalian glycoproteins.[8] Mannose-based glycosylation is associated with adverse immune response in humans. The high-mannose N-glycan-modified glycoproteins are more rapidly cleared from the bloodstream, compared to other types of glycoproteins, due to specific binding to mannose receptors which negatively affects their efficacy, pharmacokinetics, and stability as candidates for therapeutic monoclonal antibodies (mAbs). In contrast, the sialic acid glycosylation in CHO cells elongates circulatory half-life of similar proteins to improve their therapeutic efficacy.[9-12] Although therapeutic antibodies made with *P. pastoris* have been used in humans, to date,

they have only been made by removing the N-linked glycosylation sites.[13]

Over the years, synthetic biology tools have been applied to modify, or "humanize", N-glycosylation in *P. pastoris* to make it a more compatible expression system for recombinant therapeutic proteins. Such efforts include: (1) glycoengineering to eliminate hyper-glycosylation in *P. pastoris* and bring in additional glycosidases and glycosyltransferases; (2) introducing synthetic promoters to enhance expression and support desired folding; and (3) utilizing new techniques for accurate and effective genome manipulation in *P. pastoris*.[4] These efforts have led to the commercialization of human glycosylation systems including Pichia GlycoSwitch® (Research Corporation Technologies [RCT]).[14,15]

In addition, 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 popular one is the alcohol oxidase 1 (AOX1) promoter, P_{AOX1}, regulating the expression of the first enzyme in the methanol utilization pathway, AOX.[5,16] P_{AOX1} is strongly repressed in the presence of common carbon sources like glucose (*i.e.,* less than one AOX1 transcript per cell when glucose is present).[17] Upon depletion of the common carbon source, the promoter is de-repressed and capable of eliciting its full activity with the addition of methanol. For example, *Rhizopus oryzae* lipase was produced by a *P. pastoris* P_{AOX1}-based system under both methanol limited and unlimited conditions.[18] A designed analog of a spider dragline silk protein was produced at high levels by methylotrophic *P. pastoris* under P_{AOX1} with methanol induction.[19] Human serum albumin (HSA) was produced with P_{AOX1} by *P. pastoris* to show high *O*-glycosylation intensity with short linear mannose chains in fed-batch fermentation.[20] Recombinant trastuzumab (a therapeutic mAb used for the treatment of breast cancer) antibodies were produced by *P. pastoris* through methanol induction in shake flasks, and the yield of the purified recombinant trastuzumab reached 0.5 g/L.[21] Also, a soy hemoglobin is made with *P. pastoris* by Impossible Foods Inc. to serve as a sustainable source of flavor and aroma in plant-based "meats."[22] However, due to its volatility and flammability, the addition of methanol to the bioprocess system brings significant safety concerns. Therefore, alternative promoters that do not require methanol induction, yet still achieve high titers, are desired.

In collaboration with BioGrammatics, Inc. (Carlsbad, California USA), a *P. pastoris* strain was constructed with Pichia GlycoSwitch technology capable of performing more "humanized" glycosylation to produce a human immunoglobulin G (IgG) fragment crystallizable (Fc). The Fc region, composed of two identical protein fragments, undergoes specific N-linked glycosylation, which is key for specific Fc receptor-mediated activities.[23] Instead of using P_{AOX1}, the strain carries a strong constitutive promoter, P_{UPP}, which drives expression in most carbon sources including glucose, glycerol, and methanol. In this study, both biomass growth and protein expression are supported by glycerol consumption, whereas no methanol is needed.

The objective of this study is to demonstrate the feasibility of using the Pichia GlycoSwitch system (RCT) for methanol-free IgG Fc production with *in situ* mammalian-like glycosylation, which is, to our knowledge, for the first time. We believe this is an important milestone toward full-length, glycosylated, human mAb production using *P. pastoris*. The demonstration was done at bench-scale through a fed-batch fermentation.

MATERIALS AND METHODS

Yeast Strain

The Pichia GlycoSwitch SuperMan5 strains used in this study were constructed by BioGrammatics. These strains contain a human IgG Fc expression construct and a constitutive promoter P_{UPP} (BioGrammatics), also known as P_{GCW14}.[24] P_{UPP} is free of methanol and continually drives protein expression and regulation to produce heterologous protein.

Two expression vectors were created with different drug-resistant genes for selection in *P. pastoris*: pJUG-s1 and pJUN-s1. Both vectors have: (1) the P_{UPP} promoter to drive the expression of the human IgG Fc with the α-mating factor secretion signal; and (2) either the resistance gene for selection on G418 or nourseothricin N-acetyl transferase (Nat), respectively.[25] Both plasmids were prepared for electroporation into Pichia SuperMan5-10 (Pichia GlycoSwitch) by linearization in the P_{UPP} promoter with Bsu36I before the DNA was cleaned and concentrated to ~100 ng/µL in 1 mM Tris pH 7.4, 100nM EDTA. Linearization in the UPP promoter, P_{UPP}, directs integration at the native *P. pastoris* UPP/GCW14 locus by homologous recombination. Electro-competent *P. pastoris* cells were co-transformed by electroporation with 100 ng of each linear plasmid using 1 mm cuvettes at 1,150 volts, 10 µ Faraday capacitance, and 600 ohms resistance. Time constants from the electroporation were between 4–5 ms. Transformants were selected on yeast extract-peptone-dextrose (YPD)-G418 (800 µg/mL G418) and patched to YPD-Nat (80 µg/mL Nat), or vice versa, to determine if they had acquired both plasmids.

Transformants resistant to only one or both drugs were patched to selective plates after single colony isolation. Cells from these patches were used in subsequent expression testing without any drug selection.

Four SuperMan5 strains were created in total. Two of them, G1 and N1, contained a single copy of the expression vector plasmid DNA, *G418R* or *NatR*, respectively; while the other two, D1 and D2, contained two copies of the expression vector, based on strain resistance to both *G418R* and *NatR*. For all strains, the recombinant human IgG Fc was targeted for secretion by the autocrine motility factor (AMF) secretion signal in the expression vectors.

An SDS-PAGE was carried out to demonstrate the successful expression and secretion of this specific human IgG Fc from all four *P. pastoris* strains constructed through PAGE/SYPRO Ruby protein gel stain (standard per manufacturers recommendations).

Media Preparation

Two types of growth media were used in this study: YPD and buffered glycerol-complex medium (BMGY).

One liter of YPD medium contains 10 g yeast extract (Fisher Bioreagents), 20 g peptone (Bacto Proteose, Gibco), and 20 g glucose (Sigma-Aldrich). Sterile liquid medium was used when preparing the glycerol stock.

BMGY medium was prepared for bioreactor fermentation. For 1 L BMGY broth, it was made by first dissolving 10 g yeast extract, 20 g peptone, and 7.5 mL glycerol (Fisher Bioreagents) per 800 mL deionized (DI) water and autoclaving at 121°C. One hundred mL of each of the following filter-sterilized components were then added to create 1 L of BMGY: (1) 1 M potassium phosphate buffer (pH 6.0) prepared by dissolving 2.405 g K_2HPO_4 (Fisher Chemical) and 11.73 g KH_2PO_4 (Sigma-Aldrich) in 100 mL DI water, and sterilizing through 0.2 µm membrane filtration; and (2) 10X yeast nitrogen base (YNB) with ammonium sulfate, prepared by dissolving 13.4 g of the YNB powder (Invitrogen) in 100 mL DI water and sterilizing through 0.2 µm membrane filtration.

The feeding medium used in fed-batch fermentation was 50% (v/v) glycerol solution. It was sterilized by autoclave in the feeding bottle and aseptically connected to the liquid addition port on the headplate of the bioreactor before inoculation.

IgG Expression Validation, Selection, and Storage

All four *P. pastoris* strains were inoculated into 150 mL YPD medium in 500 mL Erlenmeyer flasks and incubated in a shaker (Innova® S44i, Eppendorf) at 28°C, 200 rpm agitation for 48 h. Upon completion of the shaking fermentation, all cultures turned turbid, and a 1 mL suspension from each flask was taken to pellet cells to collect the supernatant for IgG Fc analysis, using a Cedex Bio Analyzer (Roche Diagnostics). The strain with the highest IgG Fc concentration was selected for bioreactor fermentation.

For the chosen strain, 600 µL of the actively growing *P. pastoris* suspension from the shake flask was added to a cryogenic vial containing 400 µL 75% (v/v) sterile glycerol, and multiple vials were prepared. Cells were well-mixed in the cryogenic vials and transferred into a freezer at -80°C (Innova U360, Eppendorf) for future use.

Inoculum Preparation

One cryogenic vial containing the chosen GlycoSwitch SuperMan5 *P. pastoris* strain was thawed under ambient temperature and 500 µL was inoculated to a 500 mL Erlenmeyer flask containing 150 mL sterile BMGY medium (such flasks were prepared in duplicate). A third Erlenmeyer flask was set as a control, which only contained 150 mL fresh BMGY medium without inoculation. All three flasks were transferred to the shaker to grow at 28°C with 200 rpm agitation. After 48 h, the broth in the two sample Erlenmeyer flasks turned turbid from active growth of the Pichia GlycoSwitch strain. The medium in the control flask stayed clear, which proved medium sterility and aseptic manipulation.

After 48 h of shaking incubation, one flask containing *P. pastoris* suspension with the higher optical density at 600 nm (*e.g.*, OD_{600}>20) was selected to inoculate the bioreactor. OD_{600} was measured using an Eppendorf BioSpectrometer® with fresh BMGY medium as blank. Then, 75 mL of the selected suspension was poured into a 500 mL sterile liquid addition bottle to inoculate the bioreactor through pumping.

Bioreactor Setup

A fed-batch fermentation was carried out in an Eppendorf BioBLU® 3f Single-Use Bioreactor built with rigid walls and a working volume range of 1.25–3.75 L. The run started with an early-stage batch culture at 1.5 L working volume in BMGY broth until carbon source depletion. Toward the end of fed-batch, the final working volume was ~3 L. Antifoam 204 (Sigma-Aldrich) was added to the BMGY broth when filling the BioBLU 3f Single-Use Bioreactor before autoclave to reach a final antifoam concentration of 0.03% (v/v), thus 0.9 mL antifoam to a total of 3 L broth.

On the headplate of the bioreactor, two different Mettler Toledo sensors, a digital ISM® pH sensor and an analog polarographic dissolved oxygen (DO) sensor were installed through the Pg 13.5 ports for pH monitoring and

DO measurement, respectively. A stainless-steel cooling finger was installed through a compression fitting in another Pg 13.5 port. Three liquid addition ports were extended appropriately for connection with the external bottles. After autoclave sterilization, the medium-filled bioreactor was cooled to ambient temperature. Sterile 1 M potassium phosphate buffer and 10X YNB solution were added to the bioreactor in the biosafety cabinet to complete the BMGY broth preparation, as described earlier. Upon completion of the DO sensor calibration, an inoculation bottle, feeding bottle, and base bottle were aseptically connected to the liquid addition ports on the vessel headplate through a SCD®-II Sterile Tubing Welder (Terumo BCT, USA) before inoculation. The detailed setup of the BioBLU 3f Single-Use Bioreactor for microbial applications can be found in earlier studies.[26,27]

Sensor and Pump Calibration

The pH sensor was calibrated outside of the vessel before sterilization and installation. A two-point calibration method was employed by setting ZERO using buffer at pH 7.0 and setting SPAN using buffer at pH 4.0.

The pre-polarized DO sensor was calibrated after autoclave sterilization with BMGY broth in the bioreactor. Calibration was done under the same conditions as the real bioreactor culture at 28 °C, pH 6.0, and 1,200 rpm agitation. A two-point calibration method was also applied here. Nitrogen was first sparged at 1 VVM, here in this case 1.5 SLPM, until the DO value stabilized to set ZERO at 0%. Gas supply was then switched to air at the same flow rate, and SPAN was set at 100% when the DO value stabilized again.

Pump calibration was performed before bioreactor fermentation. The same tubing applied to the peristaltic pump head for liquid addition during the run was used in pump calibration. The detailed procedure for pump calibration can be found in Yang and Sha.[27]

Process Parameter Setup During Bioreactor Fermentation

The bench-scale *P. pastoris* fermentation was carried out at 28 °C, pH 6.0, and 30% DO throughout the entire culture period using a BioFlo® 320 bioprocess control station (Eppendorf). The setup is shown in **Figure 1** and the process parameters are summarized in **Table 1** (on the next page).

Good aeration is very important in the entire process. By maintaining DO at 30%, it continuously supported

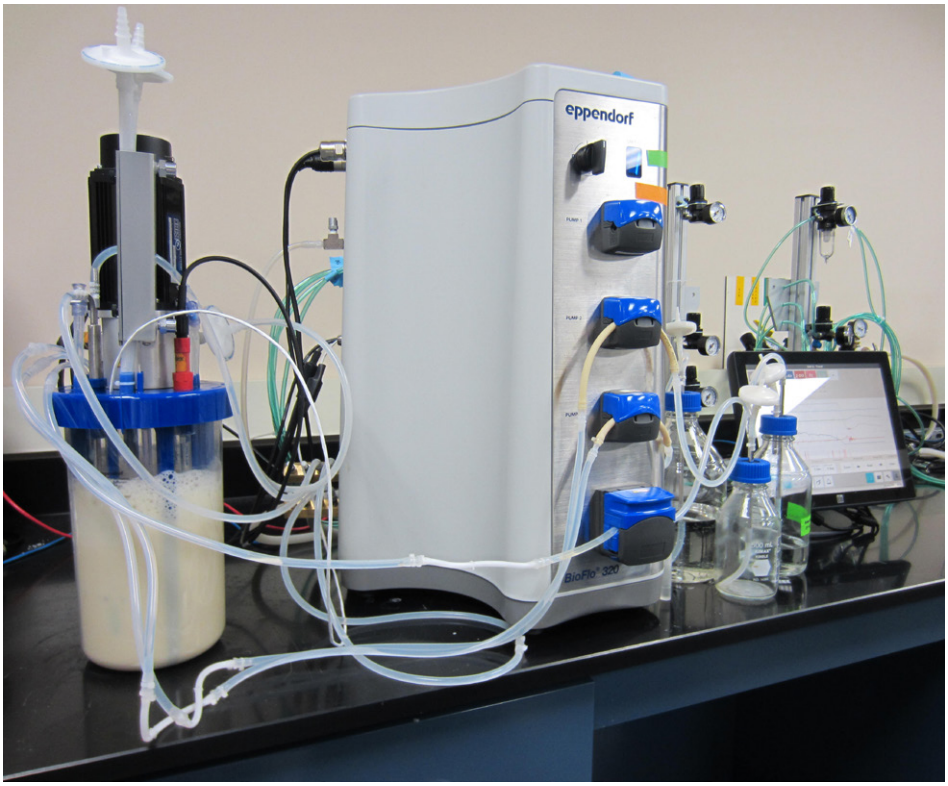

FIGURE 1. Bench-scale fed-batch fermentation of Pichia GlycoSwitch SuperMan5 strain for IgG Fc production in the BioBLU 3f Single-Use Bioreactor controlled by a BioFlo 320 bioprocess control station.

TABLE 1. Key process parameters applied to *Pichia pastoris* fermentation in the BioBLU 3f Single-Use Bioreactor controlled with a BioFlo 320 bioprocess control system.

Parameter	Configuration
Inoculation Density	5% (v/v), 75 mL inoculum to an initial 1.5 L working volume (OD_{600} = 20.2 for the inoculum used here)
Dissolved Oxygen	30%, maintained by DO cascade (as shown in Figure 2)
Agitation	Magnetic drive, maximum 1,200 rpm, controlled by DO cascade
Gassing	Automatic 4 gas flow and mix, 4 TMFC (thermal mass flow controller) at 0.04–20 SLPM, controlled by DO cascade
Temperature	28 °C, both cooling and heating are controlled by circulating water through a single stainless-steel cooling finger
pH	6.0 ± 0.1, controlled by the addition of 30% (v/v) sterile ammonium hydroxide solution
Impeller	3 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

robust aerobic yeast growth. A completely customized DO cascade was built by first accelerating the agitation from 300–1,200 rpm, then increasing the air sparging rate from 0.8–3.0 SLPM, and finally enriching oxygen in the sparged gas stream from 0–100%. These three steps corresponded to the DO output of 0–50%, 50–65%, and 65–100%, respectively. The detailed DO cascade setup in the BioFlo 320 controller is shown in **Figure 2**.

A DO spike indicated the depletion of carbon source, glycerol in this case, in the late batch stage of *P. pastoris* fermentation. Upon glycerol depletion, the consumption of oxygen and the metabolic rate of *P. pastoris* cells slowed down significantly, creating a DO spike. Here, the DO spike triggered manual operation and handling based on experience from previous runs regarding elapsed fermentation time (EFT). The feeding pump was turned on at a constant rate of 0.4 mL/min after DO spike detection on the trend page of the controller.

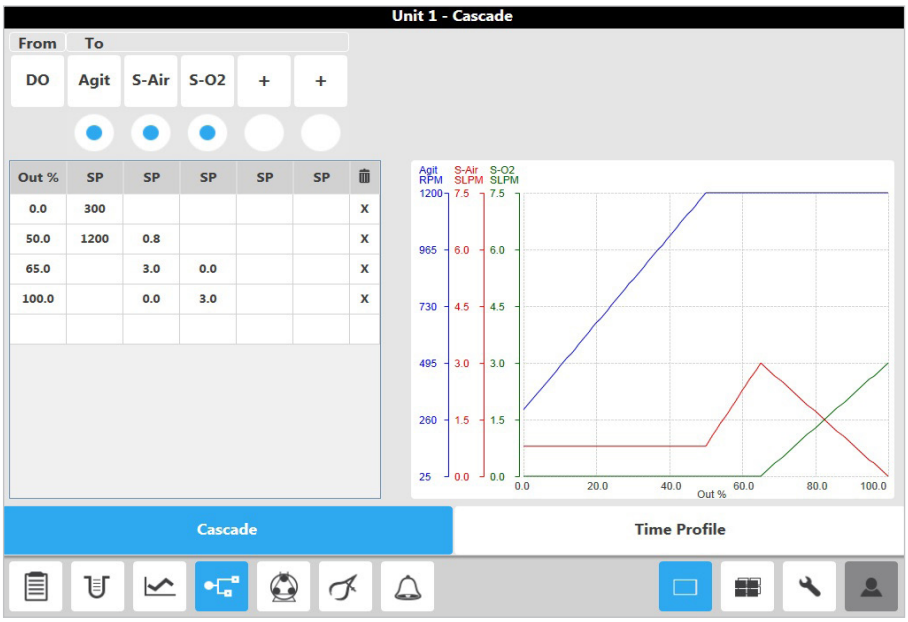

FIGURE 2. DO cascade setup in the BioFlo 320 bioprocess control station. The DO was maintained at 30% throughout the 3 L *P. pastoris* bioreactor run.

Biomass Formation – Optical Density Measurement

Upon completion of the DO calibration and right before inoculation to the bioreactor, a 30 mL sample of fresh BMGY broth was taken from the bioreactor. One mL of this medium was used to set blank/baseline for optical density measurement at 600 nm. The remaining volume was saved as the diluent for the yeast suspension collected during the run. For optical density measurement, samples were taken at nine timepoints: 0, 3, 6, 21, 24, 30, 46, 48, and 52 h after inoculation.

Human IgG Fc Analysis

Human IgG Fc was analyzed using a Cedex Bio Analyzer. Since this analyzer quantifies a full-length human IgG via Fc interactions using a protein A assay, an indirect estimate of the amount of an IgG Fc can be similarly quantified using the smaller mass of the IgG Fc. Based on the bands shown on SDS-PAGE, the molecular mass of the human IgG Fc expressed by Pichia GlycoSwitch SuperMan5 strain can be determined. Relative to the roughly 150 kDa for a full-length human IgG, the molecular mass ratio of human IgG Fc to full-length IgG can be calculated accordingly. Then, for all shake flask and bioreactor samples, such correction was applied to each reading directly from the Bio Analyzer.

Since the recombinant IgG Fc was secreted from *P. pastoris* cells, analysis was performed by collecting the supernatant after pelleting the cells from a growing culture. From each of the four Pichia GlycoSwitch SuperMan5 strains in shake flasks, 1 mL of suspension culture was taken, the cells were pelleted by centrifugation in a MiniSpin® plus microcentrifuge (Eppendorf) at 14,000 rpm for 90 s, and 500 µL supernatant was collected for IgG Fc analysis. During the 3 L bioreactor run, samples were taken at the same nine timepoints for biomass analysis, then 1 mL of supernatant was taken for IgG Fc analysis following the same procedure as for the shake flask samples.

RESULTS

Multiple Pichia GlycoSwitch SuperMan5 strains were screened to compare the expression level of IgG Fc. Its amino acid sequence is shown in **Figure 3**. The strain with the highest level, based on mAb quantification using the Bio Analyzer, was chosen for a 3 L fed-batch fermentation. Glycerol was the only carbon source used in this study; DO was maintained at 30% by the customized DO cascade; and feeding was initiated after the DO spike to avoid extended carbon source depletion. Throughout the bioreactor run, intermittent samples were taken to monitor yeast growth and production of secreted IgG Fc protein.

Pichia GlycoSwitch IgG Fc Production Strain Selection

An initial expression test of multiple clones generated from the first *P. pastoris* transformation was performed to compare strains with one or two copies of the IgG Fc expression cassette. SDS-PAGE on supernatant samples demonstrated the successful expression/secretion of the same size IgG Fc from all four *P. pastoris* strains (**Figure 4**).

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FIGURE 3. Amino acid sequence of the expressed IgG Fc with myc-6His epitope (sequence extracted from the NIH GenBank® files).

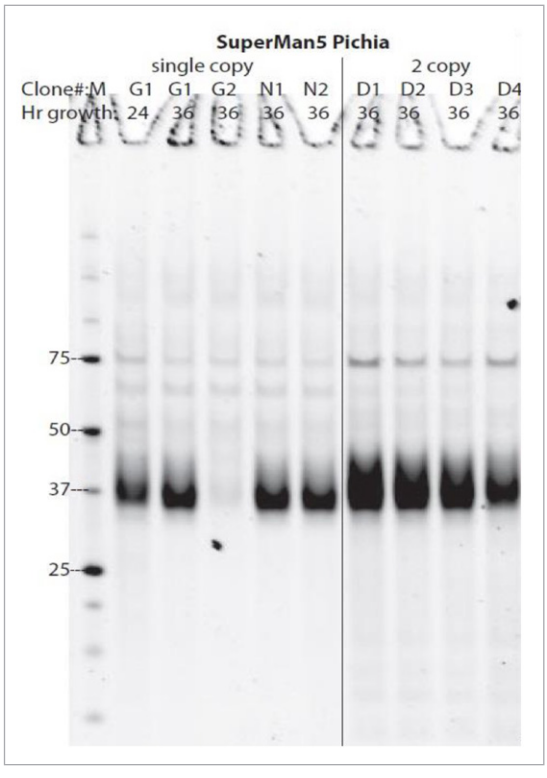

FIGURE 4. PAGE of supernatant samples from multiple clones produced with Pichia GlycoSwitch SuperMan5 strains with one and two copies of the IgG Fc expression cassette.

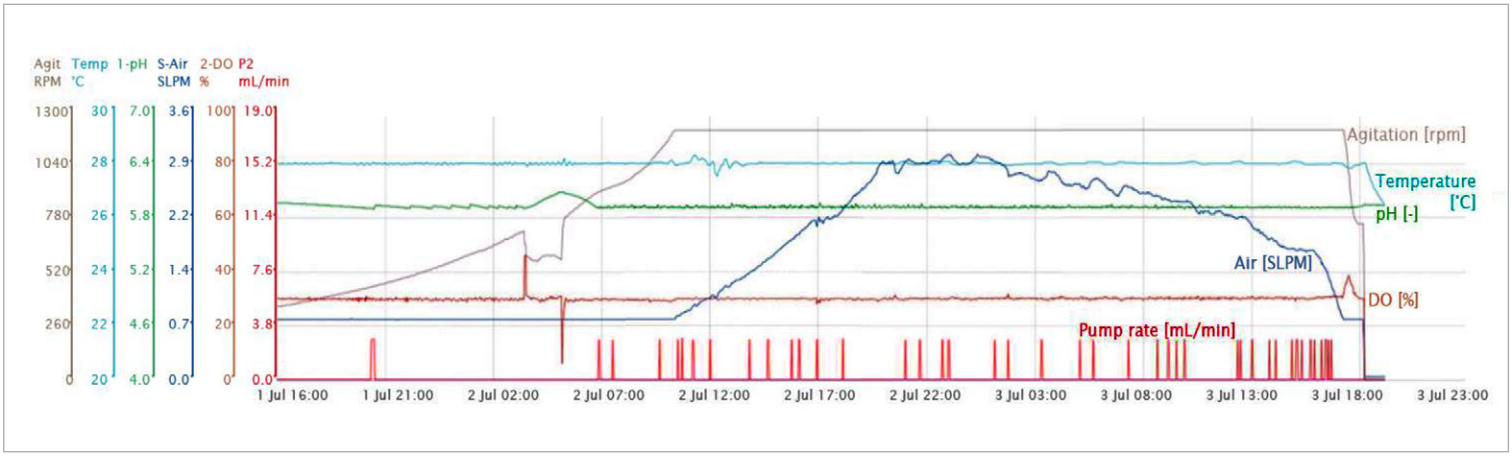

FIGURE 5. Trends from the 3 L bioreactor run. Data collected by the BioFlo 320 bioprocess control station during the 52 h Pichia GlycoSwitch SuperMan5 fermentation featuring: agitation, temperature, pH, air sparging, DO, pump activity (base addition). The figure is a combination of sequential screenshots of the trends to cover the entire culture period.

After 48 h growth in 150 mL shake flask cultures, IgG Fc concentrations were 2.2, 2.2, 3.2, and 3.2 mg/L for the G1, N1, D1, and D2 strains, respectively. The approximate molecular weight of this IgG Fc, as determined from the gel, was ~40 kDa, as predicted from the amino acid sequence. Higher levels of expression were evident from strains with two copies of the IgG Fc gene (almost two-fold). Therefore, the Pichia GlycoSwitch SuperMan5 D1 strain with two copies of the IgG Fc sequence was selected for the bioreactor run at the 3 L scale. Also, based on the molecular mass of 40 kDa, all Bio Analyzer direct readings were adjusted by multiplying 4/15 to indicate the exact concentration of human IgG Fc in the culture broth.

Bioprocess Trends Under the Control of a BioFlo 320 Bioprocess Control Station

The selected Pichia GlycoSwitch strain D1 was grown in BioBLU 3f Single-Use Bioreactor controlled by a BioFlo 320 controller. As described previously, a DO spike during the bioreactor run indicates the depletion of carbon source and is usually the signal to initiate feeding. A significant DO spike was observed at $t = 13.5$ h after inoculation. Relative to a DO setpoint at 30%, the peak of the DO spike reached 46% (**Figure 5**). Just prior to this DO spike, the culture agitation was at ~720 rpm and gradually ramping up. The DO spike was accompanied by a sharp drop of agitation rate, indicating the drastically reduced oxygen consumption when no carbon source was available. Immediately after glycerol feeding was initiated at $t = 15$ h, yeast metabolism and growth quickly resumed, causing DO to initially decrease and soon recover with agitation continuing its upward trend (**Figure 5**).

Twenty hours after inoculation, impeller agitation reached its maximum speed of 1,200 rpm and was maintained at this level for the rest of the culture. The air sparging rate ramped up from 0.8 to ~3.0 SLPM in the next 10 h and stayed at the maximum sparging rate for 30–35 h post-inoculation. No oxygen enrichment was observed, indicating that 1,200 rpm agitation and 1 VVM air sparging were sufficient to meet the largest oxygen demand of the actively growing *P. pastoris* culture with this setup. The air flow rate then started to drop from ~3 SLPM during the next 16 h, and at $t = 51$ h, air sparging was back at the minimum of 0.8 SLPM, beyond which agitation slowed down dramatically. The bioreactor run was stopped at $t = 52$ h (**Figure 5**).

As shown in **Figure 5**, throughout the entire culture period with the customized DO cascade, DO was maintained well at 30%, except during the expected DO spike at the early stage of the culture. Temperature and pH were maintained smoothly at their setpoints with effective cooling and intermitted addition of base through automated pumping, respectively.

This fed-batch bioreactor run started with a 75 mL actively growing inoculum introduced to 1.5 L fresh BMGY medium, and was supplemented by approximately 900 mL of glycerol feed solution, and 600 mL of ammonium hydroxide solution for pH adjustment. With minimum evaporation, thanks to a moderate culture temperature of 28°C and effective exhaust condensing, the final volume was ~3 L at completion.

Yeast Growth and IgG Fc Production

Yeast growth in the 3 L bioreactor was evaluated by drawing the growth curve based on the optical density

collected (**Figure 6**). *P. pastoris* displayed a relatively long lag phase before entering exponential growth. After feeding started at $t = 15$ h, robust growth continued to reach the maximum OD_{600} of 137.8 at $t = 46$ h. After that, a death phase was observed as OD_{600} started to decline at the end of fermentation.

Overall, during the 52 h bioreactor run, the growth of *P. pastoris* correlated well with the real-time oxygen demand controlled by the DO cascade. With agitation slowly ramping up to 1,200 rpm in 20 h, the *P. pastoris* culture first underwent a lag phase before initiating exponential growth. Between 20–30 h post-inoculation, the air sparging rate gradually increased to the maximum of 3 SLPM (1 VVM) and the *P. pastoris* culture displayed accelerated growth. Maximum air sparging lasted for 5 h before declining without pure oxygen supplementation to support continued biomass accumulation, and a slower specific growth rate was observed before ending in the death phase.

In the suspended culture, IgG Fc was first detected at $t = 21$ h with a titer of 24 mg/L. Beyond that timepoint, a continuous accumulation of the secreted IgG Fc was observed together with the exponential yeast growth. Maximum IgG Fc titer of 197.3 mg/L occurred at $t = 46–48$ h before it started to decline slightly during the last 4 h of the run.

DISCUSSION

Continuous feeding of glycerol effectively prevented carbon source depletion to support biomass accumulation and protein expression. Since P_{UPP} is a constitutive promoter, heterologous protein expression and secretion take place simultaneously with yeast growth. Therefore, as seen in **Figure 6**, IgG Fc titer and yeast biomass are positively correlated. This is very different from *P. pastoris* expression systems using methanol-induced promoters like the alcohol oxidase 1 promoter P_{AOX1}, which undergo biomass accumulation first and then methanol-triggered protein production. Such processes have safety concerns regarding the storage and handling of flammable methanol together with high oxygen demands to support robust aerobic culture. They also pose process development challenges regarding when to introduce methanol to shift the culture from biomass growth to protein production. However, in a methanol-free *P. pastoris* culture with a strong constitutive promoter, such as the P_{UPP} used in this study, high yields of the target protein can be obtained by simply boosting the yeast growth. Such a process is safer, shorter, more readily scalable, and easier to convert to a continuous production. *P. pastoris* expression systems with a constitutive promoter independent of methanol regulation has been successfully demonstrated for other protein production processes.[25–27]

Mammalian host cells, mainly CHO cell systems, have been the dominant workhorse for biologic production for decades, especially for mAb production. This mature, yet expensive process is recently facing many new challenges including cost-cutting demands at the large-scale pharmaceuticals production level. Prokaryotic expression systems like *E. coli* have also been widely used to produce

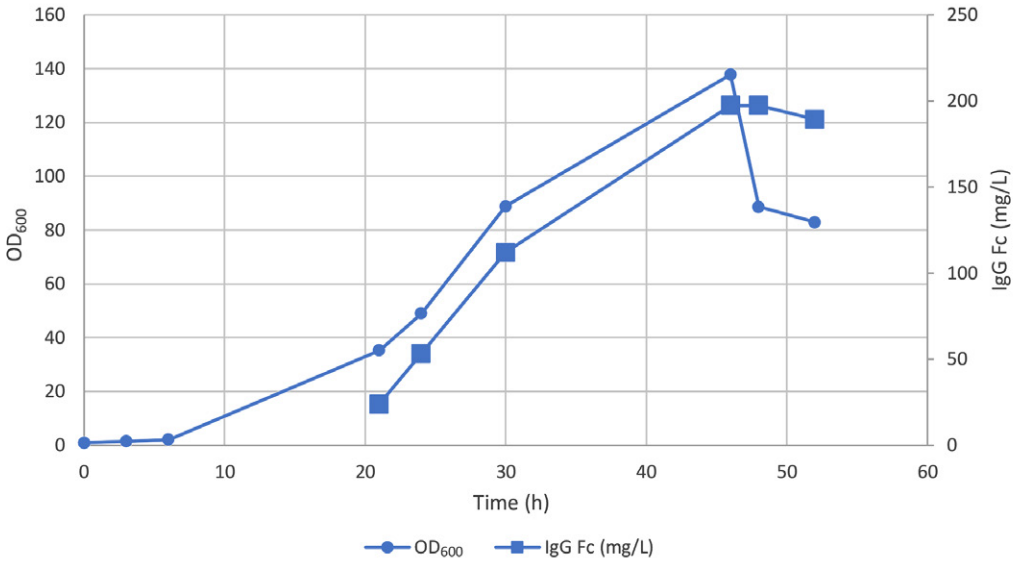

FIGURE 6. Growth curve of Pichia GlycoSwitch SuperMan5 strain and the level of secreted IgG Fc during the 3 L fed-batch bioreactor run.

smaller peptides and proteins that do not require glycosylation. As a eukaryote with short doubling time, *P. pastoris* has a long history of being the expression system for heterologous protein production with proven effectiveness over both mammalian cells and bacterial systems. However, the lack of mammalian glycosylation prevents *P. pastoris* from being used for the production of many human therapeutics. The novel Pichia GlycoSwitch System enables high expression of the desired protein with uniform, more humanized glycosylation. This now makes *P. pastoris* a competitive expression system for therapeutic protein production with much shorter culture cycles and significant cost reduction.

CHO-based mAb production is a time-proven bioprocess, typically generating the high product titers nearing 1 g/L in batch and 1–10 g/L in fed-batch cell cultures.[28] Here, a rapidly generated strain in a non-optimized 3 L fed-batch bioreactor run produced 197.3 mg/L IgG Fc. This is a 740 mg/L "full" IgG equivalent, considering the molecular weight difference between full-length IgG (150 kDa) and IgG Fc (40 kDa) expressed in this study. Thus, the Pichia GlycoSwitch strain has comparable mAb productivity to a CHO cell system, with slightly lower final titer but much shorter cultivation cycles.

Other P_{Aox1}-regulated *P. pastoris* systems for secretory recombinant protein production normally claim a protein yield ranging from milligrams to grams per liter of culture with methanol induction.[29,30] For example, Gurramkonda *et al.*[31] reported a yield of 3.1 g/L insulin precursor in the broth of a 15 L bioreactor through fed-batch fermentation; Werten *et al.*[32] presented a study on improved secretion of recombinant gelatins through fed-batch fermentation in bioreactors ranging from 1–140 L, and the 15-copy transformant was able to produce an unprecedented high yield of 14.8 g gelatin per liter of clarified broth. Therefore, *P. pastoris* is a very robust expression system for high-yield recombinant protein production. With future efforts on strain development and process optimization, this current methanol-free fed-batch bioprocess would be expected to generate a titer of IgG Fc similar to the best CHO system levels, which could potentially bring the titer of "full" IgG equivalent to be in the 1–10 g/L range at a relatively low production cost.

CONCLUSION

In summary, this study successfully demonstrates the feasibility of mAb production for diagnostics and potentially even therapeutic applications using the novel Pichia GlycoSwitch System. A relatively high titer of 197.3 mg/L human IgG Fc was produced and secreted by Pichia GlycoSwitch SuperMan5 strain in a well-controlled 3 L fed-batch culture. In addition, this was achieved in a methanol-free environment, thanks to a strong constitutive promoter, making such a process even more competitive in terms of safety and scalability. Overall, it shows that eukaryotic *P. pastoris* is potentially a competitive expression system for mAb production, which is currently dominated by mammalian cell culture, and such change of roles can trigger a new era in the biopharmaceutical industry.

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Competing Interests

The authors declare no conflict of interest.

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REFERENCES

[1] Higgins DR. Overview of protein expression in *Pichia pastoris*. *Curr Protoc Protein Sci*, 1995; Chapter 5 Unit 5.7. https://doi.org/10.1002/0471140864.ps0507s02

[2] Macauley-Patrick S, Fazenda ML, McNeil B, Harvey LM. Heterologous protein production using the *Pichia pastoris* expression system. *Yeast*, 2005; 22(4): 249–70. https://doi.org/10.1002/yea.1208

[3] Ezzine A, M'Hirsi el Adab S, Bouhaouala-Zahar B, Hmila I, Baciou L, Marzouki MN. Efficient expression of the anti-*Aah*I' scorpion toxin nanobody under a new functional form in a *Pichia pastoris* system. *Biotechnol Appl Biochem*, 2012; 59(1): 15–21. https://doi.org/10.1002/bab.67

[4] Vogl T, Hartner FS, Glieder A. New opportunities by synthetic biology for biopharmaceutical production in *Pichia pastoris*. *Curr Opin Biotechnol*, 2013; 24(6): 1094–101. https://doi.org/10.1016/j.copbio.2013.02.024

[5] Ahmad M, Hirz M, Pichler H, Schwab H. Protein expression in *Pichia pastoris*: recent achievements and perspectives for heterologous protein production. *Appl Microbiol Biotechnol*, 2014; 8: 5301–17. https://doi.org/10.1007/s00253-014-5732-5

[6] An HJ, Froehlich JW, Lebrilla CB. Determination of glycosylation sites and site-specific heterogeneity in glycoproteins. *Curr Opin Chem Biol*, 2009; 13(4): 421–6. https://doi.org/10.1016/j.cbpa.2009.07.022

[7] Dai M, Yu C, Fang T, Fu L, Wang J, Zhang J, Ren J, Xu J, Zhang X, Chen W. Identification and functional characterization of glycosylation of recombinant human platelet-derived growth factor-BB in *Pichia pastoris*.

PLOS One, 2015. https://doi.org/10.1371/journal.pone.0145419

[8] Laukens B, De Wachter C, Callewaert N. Engineering the *Pichia pastoris* N-glycosylation pathway using the GlycoSwitch technology. In: *Glyco-Engineering. Methods in Molecular Biology, vol 1321*. Castilho A ed.; Humana Press, New York, NY; 2015; pp 103–22. https://doi.org/10.1007/978-1-4939-2760-9_8

[9] Goetze AM, Liu YD, Zhang Z, Shah B, Lee E, Bondarenko PV, Flynn GC. High-mannose glycans on the Fc region of therapeutic IgG antibodies increase serum clearance in humans. *Glycobiology*, 2011; 21(7): 949–59. https://doi.org/10.1093/glycob/cwr027

[10] Nett JH, Cook WJ, Chen M, Davidson RC, Bobrowicz P, Kett W, Brevnova E, Potgieter TI *et al*. Characterization of the *Pichia pastoris* protein-*O*-mannosyltransferase gene family. *PLOS One*, 2013. https://doi.org/10.1371/journal.pone.0068325

[11] Mastrangeli R, Audino MC, Palinsky W, Broly H, Bierau H. The formidable challenge of controlling high mannose-type *N*-glycans in therapeutic mAbs. *Trends Biotechnol*, 2020; 38(10): 1154–68. https://doi.org/10.1016/j.tibtech.2020.05.009

[12] Hamilton SR, Gerngross TU. Glycosylation engineering in yeast: the advent of fully humanized yeast. *Curr Opin Biotechnol*, 2007; 18(5): 387–92. https://doi.org/10.1016/j.copbio.2007.09.001

[13] Pederson S, Biondi DM, Allan B, Cady R, Schaeffler B, Baker B, Latham J. Clinical immunogenicity evaluation of Eptinezumab, a therapeutic humanized monoclonal antibody targeting calcitonin gene-related peptide (CGRP) for the preventive treatment of migraine. *Front Immunol*, 2021; 12: 765822. https://doi.org/10.3389/fimmu.2021.765822

[14] Vervecken W, Callewaert N, Kaigorodov V, Geysens S, Contreras R. Modification of the *N*-glycosylation pathway to produce homogeneous, human-like glycans using GlycoSwitch plasmids. In: *Pichia Protocols. Methods in Molecular Biology*, vol 389. Cregg JM ed.; Humana Press, Totowa, NJ; 2007; pp 119–38. https://doi.org/10.1007/978-1-59745-456-8_9

[15] Jacobs PP, Geysens S, Vervecken W, Contreras R, Callewaert N. Engineering complex-type N-glycosylation in *Pichia pastoris* using GlycoSwitch technology. *Nat Protoc*, 2009; 4: 58–70. https://doi.org/10.1038/nprot.2008.213

[16] Gonçalves AM, Pedro AQ, Maia C, Sousa F, Queiroz JA, Passarinha LA. *Pichia pastoris*: A recombinant microfactory for antibodies and human membrane proteins. *J Microbiol Biotechnol* 2013; 23(5): 587–601. https://doi.org/10.4014/jmb.1210.10063

[17] BioGrammatics, Inc. unpublished data.

[18] Barrigon JM, Valero F, Montesinos JL. A macrokinetic model-based comparative meta-analysis of recombinant protein production by *Pichia pastoris* under *AOX1* promoter. *Biotechnol Bioeng*, 2015; 112(6): 1132–45. https://doi.org/10.1002/bit.25518

[19] Fahnestock SR, Bedzyk LA. Production of synthetic spider dragline silk protein in *Pichia pastoris*. *Appl Microbiol Biotechnol*, 1997; 47: 33–9. https://doi.org/10.1007/s002530050884

[20] Radoman B, Grünwald-Gruber C, Schmelzer B, Zavec D, Gasser B, Altmann F, Mattanovich D. The degree and length of *O*-glycosylation of recombinant proteins produced in *Pichia pastoris* depends on the nature of the protein and the process type. *Biotechnol J*, 2021; 16(3): 2000266. https://doi.org/10.1002/biot.202000266

[21] Liu C, Tsai T, Cheng T, Shivatare VS, Wu C, Wu C, Wong C. Glycoengineering of antibody (Herceptin) through yeast expression and in vitro enzymatic glycosylation. *PNAS*, 2018; 115(4): 720–5. https://doi.org/10.1073/pnas.1718172115

[22] Reyes TF, Chen Y, Fraser RZ, Chan T, Li X. Assessment of the potential allergenicity and toxicity of *Pichia* proteins in a novel leghemoglobin preparation. *Regul Toxicol Pharmacol*, 2021; 119: 104817. http://doi.org/10.1016/j.yrtph.2020.104817

[23] Peipp M, van Bueren JJL, Schneider-Merck T, Bleeker WWK, Dechant M, Beyer T, Repp R, van Berkel PHC *et al*. Antibody fucosylation differentially impacts cytotoxicity mediated by NK and PMN effector cells. *Blood*, 2008; 112(6): 2390–9. https://doi.org/10.1182/blood-2008-03-144600

[24] Liang S, Zou C, Lin Y, Zhang X, Ye Y. Identification and characterization of P_{GCW14}: a novel, strong constitutive promoter of *Pichia pastoris*. *Biotechnol Lett*, 2013; 35: 1865–71. http://doi.org/10.1007/s10529-013-1265-8

[25] Expression vector maps/sequences available from BioGrammatics, Inc. at https://www.biogrammatics.com

[26] Yang Y, Sha M. A beginner's guide to bioprocess modes – batch, fed-batch, and continuous fermentation. Eppendorf Application Note 408. 2019. https://www.eppendorf.com/product-media/doc/en/763594/Fermentors-Bioreactors_Application-Note_408_BioBLU-f-Single-Vessel_A-Beginner%E2%80%99s-Guide-Bioprocess-Modes-Batch_Fed-Batch-Continuous-Fermentation.pdf

[27] Yang Y, Sha M. From shaker to fermenter: methanol-free *Pichia pastoris* protein production workflow. Eppendorf Application Note 438. 2021. https://www.eppendorf.com/product-media/doc/en/991662/Fermentors-Bioreactors_Application-Note_438_BioFlo-Shaker-Fermenter-Methanol-free-Pichia-pastoris-Protein-Production-Workflow.pdf

[28] Kunert R, Reinhart D. Advances in recombinant antibody manufacturing. *Appl Microbiol Biotechnol*, 2016; 100: 3451–61. http://doi.org/10.1007/s00253-016-7388-9

[29] Kazemali MR, Majidzadeh K, Sardari S, Saadati AH, Barkhordari F, Adeli A, Mahboudi F, Maghsoudi A. Enhanced truncated-t-PA (CT-b) expression in high-cell-density fed-batch cultures of *Pichia pastoris* through optimization of a mixed feeding strategy by response surface methodology. *Bioprocess Biosyst Eng*, 2016; 39: 565–73. https://doi.org/10.1007/s00449-016-1538-4

[30] Liu W, Xiang H, Zhang T, Pang X, Su J, Liu H, Ma B, Yu L. Development of a new high-cell density fermentation strategy for enhanced production of a fungus β-glucosidase in *Pichia pastoris*. *Front Microbiol*, 2020; 11: 1988. https://doi.org/10.3389/fmicb.2020.01988

[31] Gurramkonda C, Polez S, Skoko N, Adnan A, Gäbel T, Chugh D, Swaminathan S, Khanna N, Tisminetzky S, Rinas U. Application of simple fed-batch technique to high-level secretory production of insulin precursor using *Pichia pastoris* with subsequent purification and conversion to human insulin. *Microb Cell Fact*, 2010; 9: 31. https://doi.org/10.1186/1475-2859-9-31

[32] Werten MWT, van den Bosch TJ, Wind RD, Mooibroek H, de Wolf FA. High-yield secretion of recombinant gelatins by *Pichia pastoris*. *Yeast*, 1999; 15(11): 1087–96. https://doi.org/10.1002/(SICI)1097-0061(199908)15:11<1087::AID-YEA436>3.0.CO;2-F

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