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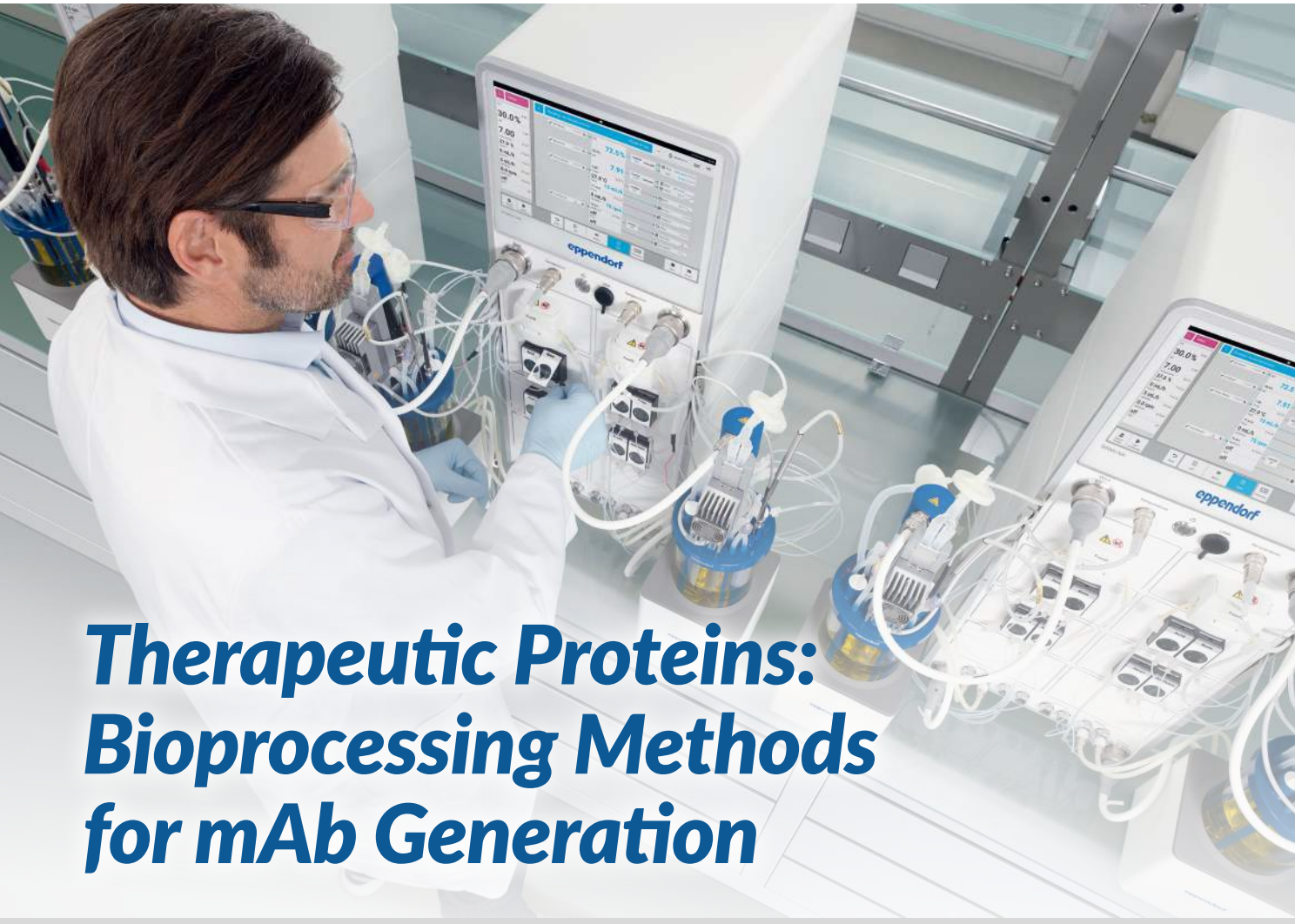

Therapeutic Proteins: Bioprocessing Methods for mAb Generation

- mAb Generation Techniques
- IgG Production via *P. Pastoris* Fermentation
- Scale-Up of CHO Antibody Production
- Effective mAb Workflows

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mAb Generation Techniques Foster R&D Advances

By Poulomi Acharya, Sherman Cheng, Pascale Galea, Yu-pin Lin, Anna Quinlan, and Mark Shulewitz

Methods for generating monoclonal antibodies for research and development.

Antibodies are key players in the body's defense against foreign agents, such as microorganisms and viruses, and have been used for research and diagnostics since the early 1900s. But for many decades their utility in the laboratory was limited by their polyclonal nature.

When confronted with a foreign agent (antigen), the human immune system generates a large number of antibodies, each of which recognizes a different part (epitope) of the antigen. This results in a vast, heterogeneous pool of polyclonal antibodies ideal for fighting off invaders but poorly suited for research and diagnostic applications that require a steady supply of antibodies that recognize the same exact epitope on an antigen.

In 1975, Koehler and Milstein overcame this major hurdle by developing a method to generate near-unlimited supplies of identical monoclonal antibodies (mAbs) that all bind to the same epitope of a given antigen.[1] This breakthrough turned antibodies into indispensable tools; mAbs are now routinely used in the laboratory, serve as diagnostics in applications that range from blood typing[2] to HIV diagnosis[3,4], and are driving a global therapeutic mAb market expected to be valued at $125 billion by 2020.[5] Here, the authors discuss commonly used mAb-generation technologies including hybridoma methods and recombinant antibody generation platforms.

USING HYBRIDOMAS TO GENERATE RESEARCH AND DIAGNOSTIC-KIT ANTIBODIES

Koehler and Milstein's groundbreaking hybridoma approach allows generation of pools of cells that secrete an unlimited supply of identical antibodies by fusing B cells from an animal host that produce a desired antibody with an immortal myeloma tumor cell line. This method can be used to generate research-grade antibodies for applications that range from Western blotting to flow cytometry as well as antibodies that act as critical reagents in diagnostic kits.

For generation of research-grade mAbs, an adaption of the traditional hybridoma approach, the mouse iliac lymph node method[6], when paired with immunization using full length antigens, and downstream assays that allow mAb specificity analysis, enables short development times and fast and efficient production of mAbs for many different applications.

To generate antibodies for inclusion in diagnostic kits, which must meet more rigorous quality standards than research-grade antibodies, a funnel-shaped screening process can be employed. Such approaches increase stringency as the thousands of clones that are often required to identify a single promising mAb proceed from one stage to the next. First, mAbs should be screened for positive reactivity with the antigen of interest and counter-screened against any relevant antigens to eliminate all cross-reacting clones; epitope mapping can be part of the screening when repertoire diversity and/or specificity are needed. Next, screening should focus on the mAb's affinity for the target antigen using assays that range from basic supernatant dilution assay to more sophisticated surface plasmon resonance techniques. Selected mAbs are then sub-cloned several times to achieve monoclonality and to eliminate clones with unstable chromosomal rearrangements. The final step is lab-scale production and purification of the mAb. This step eliminates poor mAb producers, as these clones won't be suitable for manufacturing. At the end of this process, one has in hand highly specific

and well-characterized mAbs ready for assay optimization and validation with clinical samples.

A key concern for immunoassay diagnostic kits is the obligation to maintain their performance throughout the lifetime of the commercial product. This means that mAbs included in diagnostic kits have to be secured for often more than 20 years. To ensure batch-to-batch consistency, a master cell bank must be established and the antibody stock safely stored.

GENERATING HUMANIZED ANTIBODIES FOR THERAPEUTIC APPLICATIONS

One of the main limitations of traditional hybridoma-based methods is that they rely on non-human hosts for antibody generation. This limitation can prove challenging for therapeutic applications as mouse antibodies are sometimes regarded as foreign by the human immune system, triggering the production of human anti-mouse antibodies that can damage the patient's kidneys.[7]

Several different approaches have been taken to generate antibodies that do not trigger this damaging immune response, including generating chimeric or humanized antibodies by combining mouse-generated mAb antigen binding sites with human antibody sequences[8,9] and engineering humanized mice that produce antibodies with fully human sequences. This latter approach has been used to bring several therapeutics to the market; the first therapeutic mAb generated using humanized mice, panitumumab, was approved by FDA in 2006 and several more such drugs have since been approved including nivolumab, a treatment for melanoma and squamous cell carcinoma.

RECOMBINANT ANTIBODY TECHNOLOGIES

An alternate approach, recombinant antibody generation, provides even greater control of antibody sequence and thus specificity and affinity; many platforms are completely animal-free. Recombinant antibody methods take advantage of vast libraries of synthetic antibody genes that can be easily manipulated to produce antibodies with desired specificities and affinities. These libraries are generated either using B cells from non-immunized donors or by de novo gene synthesis. Their large size (> 1 billion genes) enables selection of antibodies against virtually any antigen but also requires a new approach to identifying highly specific antibodies. Instead of screening for antibodies that bind an antigen of interest, antibodies with high affinity are identified through selection methods, such as ribosome[10], yeast, bacterial[11], or phage display[12].

By far the most popular screening method are phage display platforms, which fuse antibody heavy- and light-chain variable domains to

a phage coat protein gene.[13] The phages display this fusion protein on their surface, making it accessible for in vitro selection. During the selection process, the antigen is immobilized on a solid surface and exposed to the phage library. Phages expressing a high-affinity binding antibody interact with the immobilized antigen and are considered a specific target binder; low affinity and non-specific binders are removed during wash steps.[12] Specific binders can then be amplified in expression hosts such as *Escherichia coli*. If desired, resulting mAbs can be further manipulated in vitro to optimize binding strength, or tested and selected for optimal performance in specific assay formats.

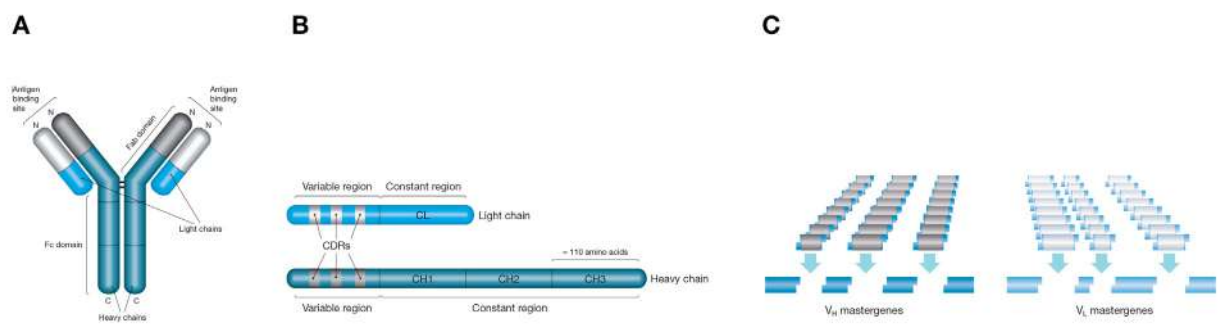
GENERATING SYNTHETIC ANTIBODY LIBRARIES

One popular approach is the generation of single-domain or VHH antibodies that consist of a single immunoglobulin domain. This technology is based on the discovery that camelid and cartilaginous fish antibodies consist only of a heavy chain (VH) domain and allows generation of much smaller antibodies that are easily manipulated in vitro and expressed in both prokaryotic and eukaryotic expression hosts, are highly stable even under extreme conditions, and have excellent penetrability for therapeutic applications. One disadvantage of these systems is that because these antibodies are of non-human origin resulting antibodies need to be humanized for therapeutic applications. The first fully synthetic Human Combinatorial Antibody Library (HuCAL) was developed by Knappik et al. in 1999.[14] Detailed analysis of the antigen binding Fv moiety of the human antibody repertoire revealed that its structural diversity can be captured by seven heavy-chain and seven light-chain variable region genes. A master library of just 49 antibody genes can thus capture the structural diversity of more than 95% of the human antibody repertoire. To generate diversity, HuCAL technology modifies the complementarity determining region (CDR) of this master library, the source of greatest variability in the antigen binding domain (FIGURE 1).

By engineering restrictions sites to flank the CDRs in the master library, synthetically engineered CDRs can be inserted to generate libraries of billions of functional human antibody specificities, which can then be selected against using in-vitro methods.[15]

Because the HuCAL library is a human antibody framework, it can eliminate the need for antibody humanization. Because it relies on swapping and randomizing CDRs in the antigen binding domain, binding affinity can be manipulated and optimized. Using guided selection methods, highly specific antibodies can be generated to recognize epitopes with specific modifications, such as phosphorylation, mutation, or oxidation. HuCAL antibodies can be engineered in various formats, including monovalent and bivalent Fab fragments that are suited for different applications. Lastly, HuCAL antibody selection and screening relies on phage-display, and high-throughput,

FIGURE 1: Generating synthetic antibody libraries. A: Schematic of an IgG molecule. B: Light and heavy chain structure. Antibody variability is driven largely by sequence heterogeneity in three short segments of the variable region., termed complementarity determining regions (CDRs). C: In the HuCAL PLATINUM (Bio-Rad Laboratories) library, the structural diversity of the human antibody repertoire is represented by seven heavy-chain and six light-chain variable region genes, which are combined to produce 42 antibody frameworks in the master library. Superimposing highly variable genetic CDR cassettes on these frameworks efffectively mimics the entire human antibody repertoire.



automated selection methods have been developed to enable the selection, screening and purification of Fab antibodies from the library in eight weeks compared to at least four months for traditional methods that rely on the immunization of animals.

IMPORTANT CONSIDERATIONS FOR USING RECOMBINANT mAbs IN RESEARCH APPLICATIONS

Unlike traditional methods, which generate mAbs in full immunoglobulin (Ig) format, recombinant technology allows generation of antibodies in non-traditional formats such as monovalent and bivalent Fab fragments with purification and detection tags, as well as functionalized antibody fragments. With the elimination of the constant FC region from the Fab antibody, nonspecific binding to Fc receptors is prevented and antibody diffusion is improved, due to their smaller size. Traditional anti-mouse and anti-rat secondary antibodies do not bind these human antibodies, so anti-human and anti-tag secondary reagents are used instead. Fab antibodies can be converted to fully human immunoglobulins of any isotype offering advantages for the design and optimization of immunodiagnostic assays. For example, human recombinant monoclonal antibodies can provide a long-term stable source of calibrator or control to replace human disease state serum, which can be variable in quality and often difficult to source consistently.

Recombinant antibodies can be easily engineered, so the format can be chosen to suit the intended application. Bivalent Fab fragments are generally preferred for applications that detect surface-bound antigens (Western blotting, flow cytometry, immunohistochemistry, etc.) because they possess two antigen binding sites, thereby increasing overall sensitivity due to avidity effects. Monovalent antibodies are ideal for crystallography and for cellular assays because they avoid cross-linking of antigens.

THE IMPORTANCE OF mAb VALIDATION

Regardless of the method of antibody generation, it is of utmost importance to validate it for the intended application. It has been demonstrated that a high percentage (> 50%) of commercially available antibodies are not specific for their intended target or are not sufficiently sensitive to detect endogenous native protein targets.[16] Various initiatives are currently underway to propose best practices for validation that could be agreed upon by antibody users in the research community and commercial antibody producers.[17] To ensure consistent and reproducible results, antibody validation should be performed for each unique assay; demonstrate that the antibody can recognize the antigen of interest in the context of those assays; and include negative and positive controls whenever possible to demonstrate specificity of the antibody for the antigen.

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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, *P. 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.

MATERIAL 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, 100 nM 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.

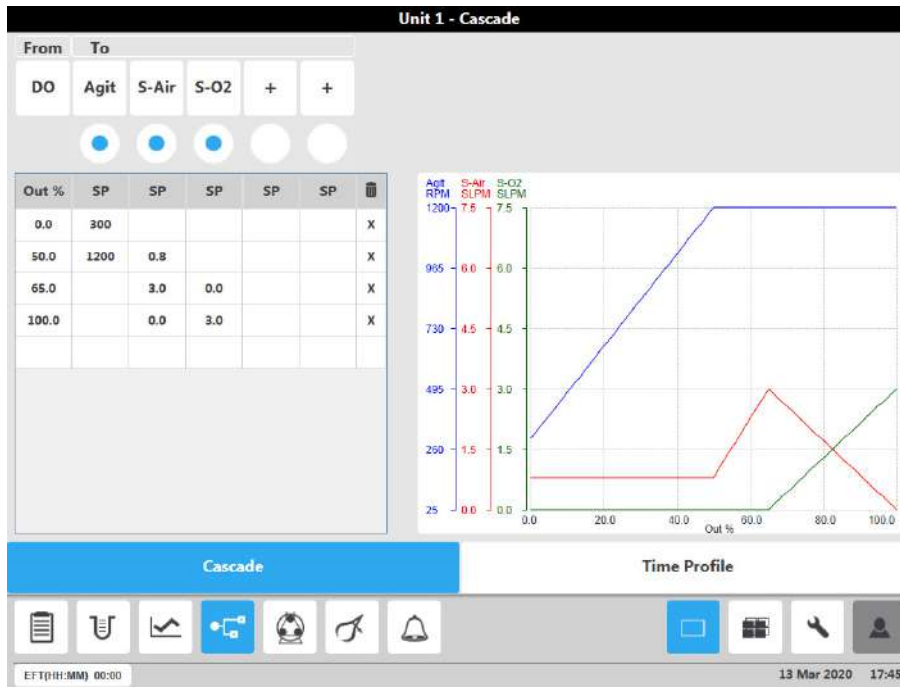
Good aeration is very important in the entire process. By maintaining DO at 30%, it continuously supported robust aerobic

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 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 (DO)	30%, maintained by DO cascade as shown in Fig. 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	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

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.



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.

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.

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

/translation=
"EFEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPE
VTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTY
RVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPR
EPQVYTLPPSREEMTKNQVSLTCLVKGFYPSD IAVEWESNGQPE
NNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEA
LHNHYTQKSLSLSPGKAAASFLEQKLISEEDLNSAVDHHHHHH"

RESULTS

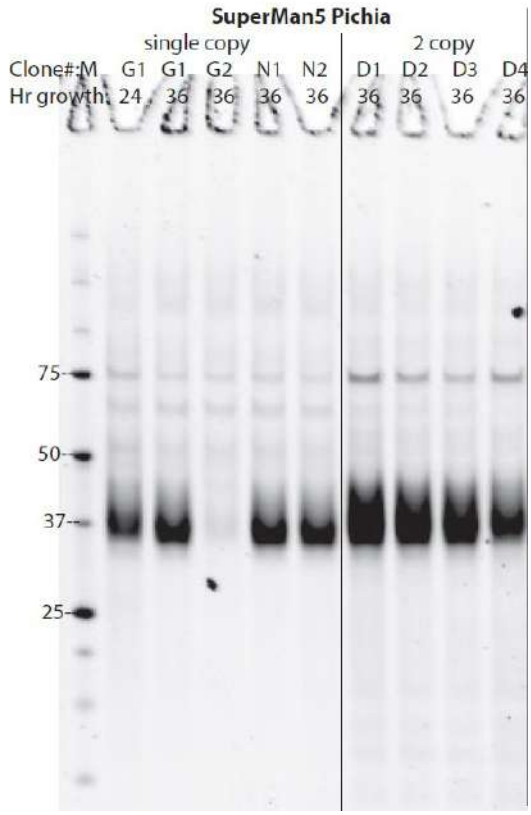
Multiple Pichia GlycoSwitch SuperMan5 strains were screened to compare the expression level of IgG Fc. Its amino acid sequence is shown in FIGURE 3. The strain with the highest level, based on mAb quantification using the Bio Analyzer, was chosen for a 3 L fed-batch fermentation. Glycerol was the only 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).

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.

FIGURE 4: PAGE of supernatant samples from multiple clones from Pichia GlycoSwitch® strains with 1 and 2 copies of the IgG Fc expression cassette.



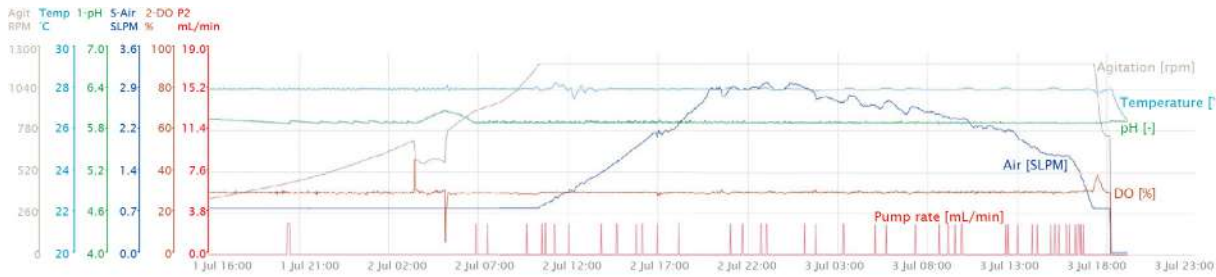
Bioprocess Trends Under the Control of a BioFlo 320 Bioprocess Control Station

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

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.



and pH were maintained smoothly at their setpoints with effective cooling and intermitted addition of base through automated pumping, respectively.

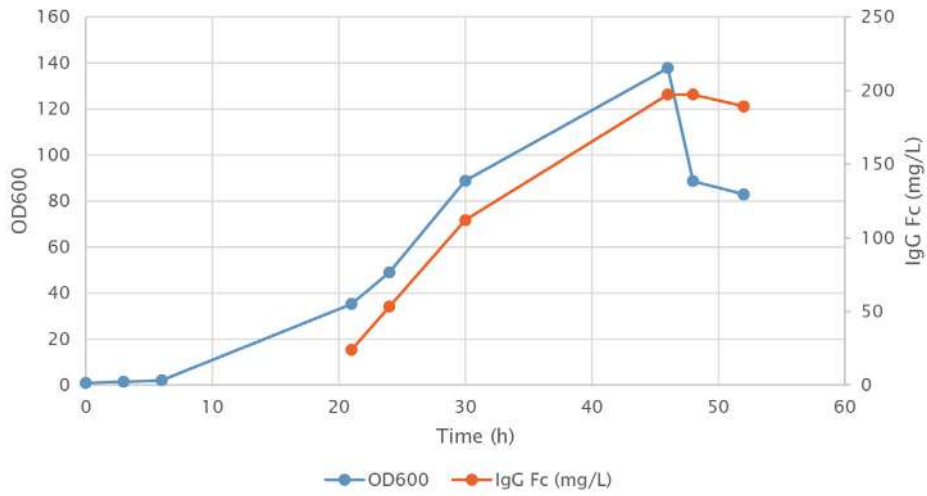
This fed-batch bioreactor run started with a 75 mL actively growing inoculum introduced to 1.5 L fresh BMGY medium, and was supplemented by approximately 900 mL of glycerol feed solution, and 600 mL of ammonium hydroxide solution for pH adjustment. With minimum evaporation, thanks to a moderate culture 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.

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



In the suspended culture, IgG Fc was first detected at t = 21 h with a titer of 24 mg/L. Beyond that time¬point, 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 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.

STATEMENTS AND DECLARATIONS

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Competing Interests

The authors declare no conflict of interest.

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Human IgG Fc Production Through Methanol-Free Pichia pastoris Fermentation. By Ying Yang, PhD, Ma Sha, PhD, and Knut Madden, PhD. BioProcessing Journal, Vol. 21. Republished with permission (© BioProcessing Journal). https://doi.org/10.12665/J21OA.Yang

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Scale-up of CHO Culture-Based Antibody Production from BioBLU® 3c to BioBLU® 50c Single-Use Bioreactor

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ABSTRACT

The complexity of developing new generations of therapeutics, enabling increasingly personalized treatments, is growing rapidly and faces major challenges from a manufacturing perspective. That is why an effective scale-up strategy is required to implement a reliable process and ensure reproducible yield at large working volumes without additional optimization. This study details the use of a combination of a single SciVario® twin bioreactor control system and BioBLU® 3c and BioBLU 50c Single-Use Bioreactors as a model platform for efficient bioprocess scale-up.

Specifically, we cultured Chinese hamster ovary (CHO) cells and produced antibodies in a BioBLU 3c Single-Use Bioreactor (3 L working volume) for 3 days and used its content as inoculum to scale up the CHO culture to a BioBLU 50c Single-Use Bioreactor with 30 L working volume, while both culture runs were controlled by the same SciVario twin bioreactor control system. We monitored and analyzed metabolites, cell density and viability twice daily, and achieved a robust cell growth (peak density around 15 × 10^6 cells/mL) in line with previous applications.[1]

INTRODUCTION

Manufacturing the next generation antibody therapeutics requires flexible and rapid pilot scale adaptation which always presents a challenge for companies seeking to meet product demand. In this sense, a predictable scale-up process is a good strategy to increase the production volume on a commercial scale.[2]

BioBLU Single-Use Bioreactors combine the advantages of single-use technology with the trusted performance and scalability of a stirred-tank design, eliminating labor-intensive cleaning and enhancing product safety by reducing the contamination risk.

Furthermore, the combination of BioBLU Single-Use Bioreactors and the SciVario twin bioreactor control system creates an ideal toolset for bench to pilot process scale-up with one system, reducing time and investment needs for additional bioprocess equipment.[3–5]

By controlling two bioreactors sequentially or in parallel, it is possible on the one hand to obtain a healthy and robust inoculum in a smaller scale bioreactor (using one of the two sides of the SciVario twin unit) and on the other hand to inoculate another bioreactor (located on the other side of the controller unit).

With the SciVario twin, such a self-scale-up process is possible in a range from 0.2 L to 40 L and has the advantage of precisely controlling and comparing the process parameters amongst varying scales.

It is possible to maintain parameters such as pH, DO, temperature at the desired setpoints during the inoculation process, avoiding any cell culture lag phase after inoculating a new bioreactor and achieving high cell density and viability.

In this study, our main goal was to demonstrate the feasibility of scaling up 3 L of CHO culture to 30 L, using the BioBLU 3c and 50c Single-Use Bioreactors without using multiple controllers or managing turnover with multiple runs. We analyzed the cell growth, viability, and metabolic activity (levels of glucose, lactate, and ammonia in the medium) in both bioreactors.

MATERIAL AND METHODS

SciVario twin and BioBLU Single-Use Bioreactors

In these experiments, the SciVario twin bioreactor control system was employed to scale up from 3 L to 30 L using BioBLU

3c and BioBLU 50c Single-Use Bioreactors equipped with a single pitched-blade impeller. Each bioreactor control system is equipped with three universal port connectors for pH and Dissolved Oxygen (DO) sensors, a heat blanket, agitation control and a gas module that includes a TMFC (Thermal Mass Flow Controller) with standard gas flow rates of 0.1 – 1,200 sL/h (resulting in an ultra-high turndown ratio of 1:12,000), as well as four solenoid valves (FIGURE 1).

FIGURE 1: The SciVario twin bioreactor control system allows the control two glass or single-use bioreactors, either individually or in parallel, at the same time across a wide range of vessel sizes from small- to bench-scale. It was developed for both cell culture and microbial fermentation applications.

Cell line and medium

For all experiments, a proprietary suspension CHO cell line (capable to produce a human monoclonal antibody (hmAb) from TPG Biologics, Inc., was culture in Dynamis AGT Medium (Thermo Fisher Scientific®). The medium was supplemented with 8 mM Gibco® GlutaMAX (Thermo Fisher Scientific), 1% Antibiotic Antimycotic solution (Thermo Fisher Scientific), and 1% Gibco Anti-Clumping Agent (Thermo Fisher Scientific) for a complete medium.

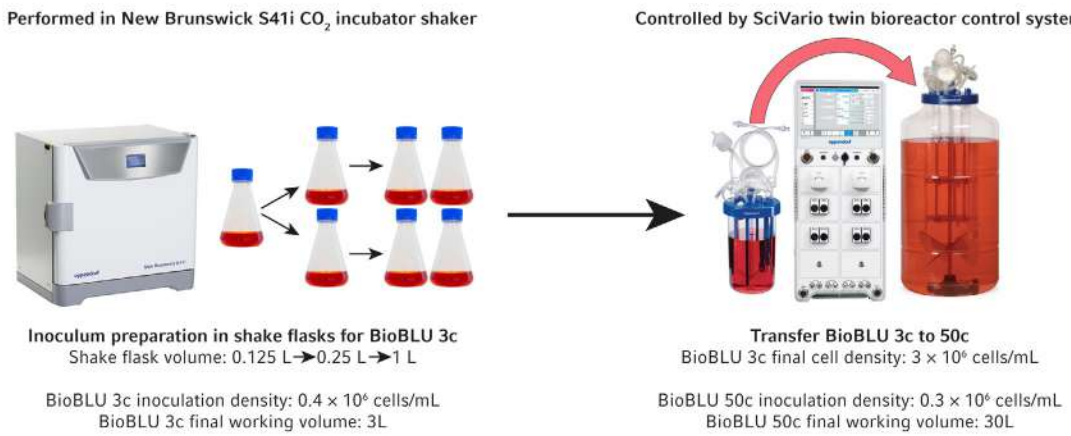
CHO cell scale-up procedure

After thawing, the cells were subjected to routine passaging and seed train or flask culture scale-up (FIGURE 2). Hereafter, the bioreactor process using the SciVario twin bioreactor control system in combination with the BioBLU Single-Use Bioreactors was initiated (FIGURE 2). For this study, a batch run was performed. Each step of the process is further described in more detail below.

Sensor calibration

Prior to the preparation of the BioBLU 3c and 50c Single-Use Bioreactors, the ISM® gel-filled pH sensors (MettlerToledo®) were connected to the SciVario twin bioreactor control system through one of the universal ports where they were automatically detected by the software. Calibration was performed according to the operations manual using known buffer solutions of pH 7 as “zero” and pH 4 as “span” respectively. Thereafter, the pH sensors were disconnected from the controller and sterilized in an autoclavable pouch.

FIGURE 2: Overview of CHO cells scale-up process.



BioBLU 3c and BioBLU 50c Single-Use Bioreactors preparation and process parameters

The BioBLU 3c and BioBLU 50c Single-Use Bioreactors were equipped with magnetic drives. The sterilized pH sensors were inserted into a spare PG 13.5 port under aseptic conditions in a biosafety cabinet. In addition, the polarographic DO sensors (Mettler Toledo®) were inserted into a non-invasive sensor sleeve inside the bioreactors. DASGIP® Peltier exhaust condensers were connected to each bioreactor and the sparge line of the bioprocess controller was connected to the sparge filter. Three liquid addition ports were used on each bioreactor: one for inoculation/medium addition, one for base addition and another for the addition of the 0.1 % of antifoam reagent (Sigma-Aldrich® Antifoam C Emulsion, Merck). Then, the heating blankets were fitted tightly around the BioBLU Single-Use Bioreactors to ensure uniform heat supply. Finally, each bioreactor was filled with Dynamis AGT complete medium and conditioned for at least 24 hours applying the process parameters and setpoints listed in TABLE 1.

TABLE 1: Process parameters and setpoints of the batch culture experiments.

	BioBLU 3c	BioBLU 50c
Working volume	3 L	30 L
Agitation	174 rpm (tip speed 0.6 m/s)	69 rpm (tip speed 0.6 m/s)
Temperature	37 °C	
Inoculation density	0.3-0.4 × 10⁶ cells/mL	
Cell culture medium	Dynamis AGT complete medium	
DO setpoint	50 % (P = 0.1; I = 3.6/h)	
pH setpoint	7.0 (deadband = 0.1), cascade to CO_2 (acid) cascade to 0.45 M sodium bicarbonate (base)	
Gassing range	0.1 SLPH – 60 SLPH	0.1 SLPH – 450 SLPH
Gassing cascade	Set O_2 % at 30 % controller output to 21 % and at 100 % controller output to 100 %. Set flow at 0 % controller output to 0.1 SLPH, and at 100 % controller output to 60 SLPH	Same as BioBLU 3c, except the maximum gassing limited to 450 SLPH

BioBLU 3c Single-Use Bioreactor inoculum preparation

Initial cell expansion was carried out in single-use, baffled bottom shake flasks (Corning®) with 20% maximum fill volume. For that, the cells were thawed from a cryopreserved stock vial and seeded into a 125 mL flask at a seeding density of 0.3 × 10^6 cells/mL. The cells were passaged every other day. After monitoring cell growth and viability (determined to be >95 %), the culture volume was stepwise increased from 125 mL to 250 mL, and finally 1 L shake flasks. During this scale-up step, the cells were cultured in a New Brunswick S41i CO_2 incubator shaker at 37°C, 8% CO_2 and 125 rpm agitation speed. Some parameters like flask inoculation density, percentage fill among others remained constant. More than 1.5 × 10^9 cells were obtained from the 1 L shake flasks. 1.2 × 10^9 of these cells suspended in 200 mL Dynamis AGT complete medium were subsequently used to inoculate the BioBLU 3c Single-Use Bioreactor for a final working volume of 3 L (initial inoculation cell density: 0.3 × 10^6 cells/mL).

BioBLU 50c Single-Use Bioreactor inoculum preparation in the BioBLU 3c Single-Use Bioreactor

In order to obtain the inoculum for the larger 30 L run within the BioBLU 50c Single-Use Bioreactor, a 3 L CHO cell culture was grown in a BioBLU 3c Single-Use Bioreactor as described in "BioBLU 3c Single-Use Bioreactor inoculum preparation". Once the cell density in that culture set-up reached approximately 3 × 10^6 cells/mL (cell viability >95%), the harvest line of the BioBLU 3c Single-Use Bioreactor was welded onto the harvest line of the BioBLU 50c Single-Use Bioreactor to facilitate inoculation of the larger bioreactor (initial inoculation cell density: 0.3 × 10^6 cells/mL). Incubation parameters were maintained as described in TABLE 1 and 0.1% Sigma-Aldrich® Antifoam C Emulsion (Merck) was added as needed.

Sampling and analytics

Samples were collected twice a day from each bioreactor to determine cell viability, cell density, and the concentration of the metabolites glucose, lactate, and ammonia (NH_3). For that, a sterile 5 mL syringe was connected to the Luer Lock sample port. Three mL of dead volume were discarded before collecting 3 mL of the culture for analysis in a new sterile 5 mL syringe. Cell density and viability were measured in a Vi-CELL® XR Viability Analyzer (Beckman Coulter®) via the trypan blue exclusion method. pH values were analyzed offline by using an Orion Star 8211 pH-meter (Thermo Fisher Scientific). The resulting pH value was used to re-standardize the pH calibration on the controller daily in order to prevent any discrepancy between online

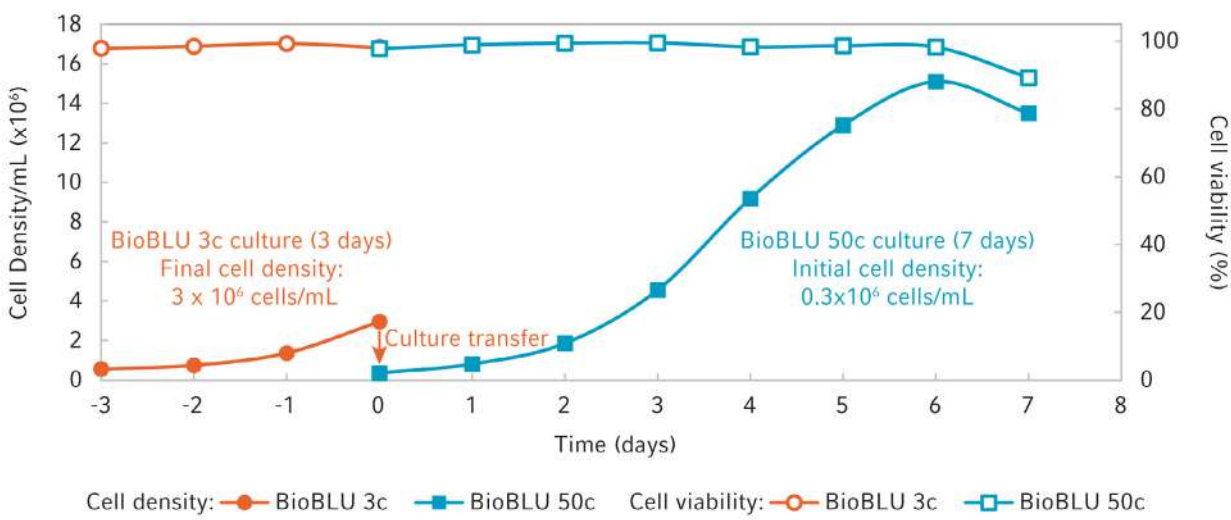
and offline measurements. Glucose, lactate, and ammonia levels were identified by using a CEDEX® Bio Analyzer (Roche Diagnostics®).

RESULTS AND DISCUSSION

The purpose of this application was to demonstrate the feasibility of the SciVario twin bioreactor control system and BioBLU Single-Use Bioreactors for CHO cell self-scale-up. The versatility of the SciVario twin allows for the operation of two processes using bioreactors of different sizes from small scale (1 L) to bench/pilot scale (up to 40 L).

Inoculum preparation for the 30 L run was performed for three days in a BioBLU 3c Single-Use Bioreactor with a working volume of 3 L. During this time cell viability remained >95% and a steady increase in cell density was observed, reaching 3 × 10^6 cells/mL on the third day of incubation (FIGURE 3). Subsequently, the whole culture volume was used to inoculate a BioBLU 50c Single-Use Bioreactor to a final working volume of 30 L with a starting cell density of 0.3 × 10^6 cells/mL. With the inoculum being within the logarithmic growing phase, cell density within the BioBLU 50c Single-Use Bioreactor increased steadily, peaking at 15 × 10^6 cells/mL on day 6 of incubation while maintaining viability levels of >95% (FIGURE 3). This peak was followed by a decrease in cell density and viability on day 7.

FIGURE 3: CHO cell density and viability during the BioBLU 3c and 50c Single-Use Bioreactor batch runs.



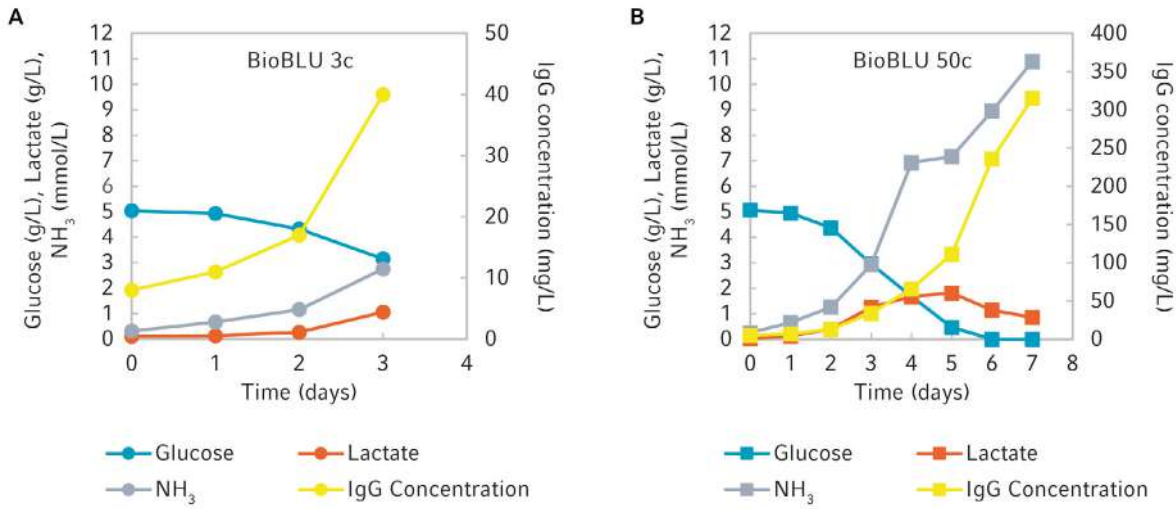
Throughout our experiments, metabolite concentration was monitored twice a day. Lactate levels remained under 2 g/L during the run. However, the actively growing culture depleted the initially supplied glucose by day 6 of incubation while ammonia concentration rose to toxic levels of up to 11 mmol/L on day 7 (FIGURE 4B). Thus, the halt of growth between day 6 and day 7 of incubation can probably be attributed to nutrient deprivation and toxic by-product accumulation, both typical phenomena in elongated batch cultivation. Similar tendencies towards decreasing glucose and increasing ammonia levels were also visible during the inoculum preparation within the BioBLU 3c Single-Use Bioreactor (FIGURE 4A). However, as this bioprocess run was terminated earlier to use the culture for the BioBLU 50c Single-Use Bioreactor inoculation, the effect on cell density did not manifest in this setting.

Despite the natural limitations of batch cultivation, a steady increase of IgG antibody expression was shown for both bioreactor cultures over the course of the experiment (FIGURE 4A/B). We would like to highlight that the metabolic concentration levels in the BioBLU 3c Single-Use Bioreactor inoculum were optimal to ensure a perfect inoculation and a successful run (FIGURE 4A).

CONCLUSION

In this study, we demonstrated a scale-up process using a single SciVario twin bioreactor control system connected to two

FIGURE 4: Metabolic profile and antibody production. A: BioBLU 3c Single-Use Bioreactor. B: BioBLU 50c Single-Use Bioreactor.



different BioBLU Single-Use Bioreactors. The SciVario software allows users to run small to bench/pilot scale bioreactors with volumes ranging from 0.2 to up to 40 L. The set-up used here with two bioreactors simultaneously connected to one SciVario twin bioreactor control system enabled efficient preparation of a 3 L inoculum in one vessel (BioBLU 3c Single-Use Bioreactor) and transfer of that inoculum under sterile conditions to another larger vessel (BioBLU 50c Single-Use Bioreactor) with a working volume of 30 L.

The direct inoculum-transfer from one bioreactor to the next by connecting their harvest ports strongly decreases the contamination risk and the use of one control system for two reactors reduces equipment requirements. The efficient and simple configuration of SciVario twin bioprocess control system allows precise control of the inoculum environment during the inoculation process leading to a rapid CHO cell growth for 6 days. These experiments were conducted primarily for feasibility demonstration. Still, we consider this approach as a helpful starting point for future scale up applications using the SciVario twin bioreactor control system.

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Monoclonal antibody (mAb)-based immunotherapy is a dominant and growing industry producing a promising class of therapeutics such as targeted anticancer drugs. However, satisfying market needs with competitive products entails several hurdles and risks. How can manufacturers tackle the challenges?

When it comes to a fast time-to-market or staying ahead in the market, time is money. The early stages of cell-line development and scale up are key to the overall success of the process.

Establishing a highly robust, efficient, and cost-effective mAb workflow requires an early processes characterization. Reproducible, reliable, and stable processes must be developed, featured by reproducible cell growth, consistent high viability of cells, and maximized yield. Highest efficiency counts, benchmarked with reduced manual handling steps and fast workflows. Single-use technologies and process-intensification help address these goals.

Employing continuous manufacturing strategies results in higher yields and can help to reduce the costs, compared to classical batch and fed-batch approaches. A comparable transfer from small scale to larger volumes is the prerequisite for a fast time to market, to mitigate risks of supply chain and market access.

PROCESS DEVELOPMENT – MAXIMIZING YIELDS AND PROCESS STABILITY

Challenge and goal are always maximizing yield, which depends on the viability and productivity of the cells. And nothing is exactly the same. Each cell line and even each clone performs differently in bioreactor conditions, such as mixing or oxygen mass transfer. To ensure stable cell growth profiles and antibody yields from small- and bench-scale to production-scale requires an effective strategy based on a detailed process understanding and optimized reactor conditions.

There are many straightforward and innovative ways to achieving this. Media optimization, cloning techniques, designing a better expression system, or studying the metabolic pathways can increase productivity. Precise equipment such as pumps, sensors, and gas control is the prerequisite to ensure stable and consistent process runs. Proper instrumentation allows to monitor metabolic pathways, assessing productivity consequences of, for example carbon source depletions, and optimize conditions in the reactor.

Analytical equipment with sensor integration in parallel systems is superior for process insights. Different settings, parameters and cell lines can be tested at early stage at the same time. The parallel control of multiple small-volume bioreactors and simultaneous monitoring of various process parameter speeds up process characterization and increases time and cost efficiency. Advanced bioprocess control systems offer the possibility to calibrate different sensors (e.g., DO, pH) and pumps in parallel, resulting in additional time-savings. An automated process control with sensor-driven feedback loops enables automation, reduces manual handling steps, the risk of human errors and contaminations, thereby increasing

safety and reproducibility. Remote process and device monitoring with alerts and notifications allows for permanent control from anywhere and timely action in case of critical variances or emergencies.

Strict contamination controls are essential to avoid batch failure. This starts with thawing cells not manually or in a water bath, but with specialized equipment. It ends with using the pumps of the bioprocess controller to inoculate the next larger bioreactor without the need of direct human interactions.

SCALE UP - MAINTAIN CELL GROWTH PROFILES AND ANTIBODY YIELDS ACHIEVED AT BENCH SCALE

One of the major mAb production challenges is cell culture scale-up. Studying the reactor conditions like evaluating the consistency of heat-transfer or finding the optimal RPM help cells grow and divide effectively and to produce a proper amount of product.

Popular scale up strategies include constant tip speed, constant impeller power per unit volume (P/V), or matching oxygen volumetric mass transfer coefficients (k_La). They enable a reproducible transfer from small scale to larger volumes and aim to maintain the cell growth profiles and antibody yields achieved at bench scale. However, calculations are complex and require expert knowledge. Essential for efficient mixing and mass transfer throughout the different bioreactor sizes is an optimal vessel geometry based on the ratio of impeller diameter to vessel diameter.

State-of-the-art software promotes in-process control with fewer manual steps. Wizards guide quickly and step-by-step through the process. Software guided calculations of important process parameters save time, considerably ease process setups, and mitigate the risk of failures. Combined with new open-concept controllers, similar cell growth profiles can be produced in a variety of bioreactors, regardless of supplier, from bench to production scale.

DEVICE MANAGEMENT – PREVENTING DOWNTIMES AND FAILURES

Cutting corners in selecting equipment could have a big and potentially negative impact on the process. The worst-case scenario leads to batch failures that cause supply shortages of critical medication.

Systems should support industry standards, give insights about its status, and send reminder if service is required. Industries with a large base of installed equipment will particularly benefit from that. Digital sensors that provide information about

their lifetime allow to replace a sensor that is close to end-of life. This can lead to significant cost savings.

Instrumentation suppliers should provide any data on the suitability of the equipment for a specific process. Being familiar with problems manufacturers face, they can provide expert support based on a common understanding. Employee training, and regular re-training in all areas of the process, is best-practice because the human element is the biggest variable when it comes to success.

Development of a robust, efficient, and cost-effective mAb research and development process is accompanied by many hurdles and risks. With the right and reliable equipment, process development and optimization at bench, pilot and production scale will lead to success.