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ORIGINAL PAPER

High-throughput screening and selection of yeast cell lines expressing monoclonal antibodies

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Abstract The methylotrophic yeast *Pichia pastoris* has recently been engineered to express therapeutic glycoproteins with uniform human N-glycans at high titers. In contrast to the current art where producing therapeutic proteins in mammalian cell lines yields a final product with heterogeneous N-glycans, proteins expressed in glycoengineered *P. pastoris* can be designed to carry a specific, preselected glycoform. However, significant variability exists in fermentation performance between genotypically similar clones with respect to cell fitness, secreted protein titer, and glycan homogeneity. Here, we describe a novel, multidimensional screening process that combines high and medium throughput tools to identify cell lines producing monoclonal antibodies (mAbs). These cell lines must satisfy multiple selection criteria (high titer, uniform N-glycans and cell robustness) and be compatible with our large-scale production platform process. Using this selection process, we were able to isolate a mAb-expressing strain yielding a titer (after protein A purification) in excess of 1 g/l in 0.5-l bioreactors.

Keywords Antibody · Yeast · Screening · *Pichia pastoris* · Fermentation

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Introduction

Recombinant proteins produced in a variety of microbial and mammalian expression systems constitute a very successful and highly innovative class of biotherapeutic agents. Since the launch of the Orthoclone OKT3 by Johnson & Johnson in 1986, monoclonal antibodies (mAbs) have emerged as the most attractive biomolecules for several clinical applications including oncology, arthritis, immune disorders and infectious diseases. In 2006 the global sales of mAbs reached a record $20.6 billion, and this commercial success has been described as the monoclonal antibody "Gold Rush" era [1]. Hundreds of new antibody development programs necessitate a significant increase in manufacturing capacity to satisfy the demand for clinical materials and commercial supply.

The current mAb production technologies employ mammalian cell lines such as Chinese hamster ovary (CHO) and mouse lymphoid cells (e.g. NS0 and SP2/0-Ag14) [2]. Recent progress in achieving stable expression from these systems combined with improvements in culturing methods and in downstream processing have resulted in high mAb titers, in excess of 1 g/l [3]. Nonetheless, the increased demand for mAbs and other therapeutic proteins combined with the pressure to reduce process development cost and cycle time has resulted in the development of alternative protein production systems including bacteria, yeast and filamentous fungi, insect cells, and plants [4–7]. In contrast to mammalian cell lines, microbial and yeast systems can offer intrinsic advantages such as ease of genetic manipulation, stable expression, adequate protein titers, rapid cell growth, and low-cost scalable fermentation processes.

Yeast species combine all the advantages of microbial systems described above with the ability to perform

eukaryote-specific post-translational modifications. Consequently, they have been widely used in biotechnology for many applications including protein production [8]. Over the last decade several yeast species have been developed into production host cells. These include *Saccharomyces cerevisiae*, *Kluyveromyces lactis*, *Hansenula polymorpha* and *Pichia pastoris* [9–12]. *Pichia pastoris* is a methylotrophic yeast with a proven record as an excellent host for the intra- and extracellular expression of over 400 different proteins, including human proteins of therapeutic value [13]. The *P. pastoris* expression system offers a strong, tightly regulated, inducible promoter (from the alcohol oxidase I gene) along with the ability to grow to high cell densities in a well-established fed-batch fermentation process [9]. *Pichia pastoris* cultures are free of both endotoxin present in bacterial systems and potential viral contaminations associated with mammalian cell cultures.

Despite these several obvious advantages, *P. pastoris* and other yeast species are rarely considered for the production of therapeutic glycoproteins because of the immunogenic nature of the nonhuman fungal glycan structures. Like other yeast species, *P. pastoris* cells produce glycans of high mannose type [14]. Additionally, the presence of nonhuman glycans can negatively affect the pharmacokinetic characteristics of therapeutic glycoproteins including mAbs. To overcome this limitation, the *P. pastoris* protein glycosylation pathway has been recently reengineered to produce fully human glycans [15–18]. The availability of this novel yeast-based expression system capable of performing human glycosylation presents the unique opportunity to dramatically impact antibody development programs and industrial production of mAbs. In addition, glycoengineered *P. pastoris* strains allow unprecedented control over the glycosylation pattern and homogeneity of the final product. This in turn can be used to enhance the clinically relevant properties of glycoproteins [5]. For example, when expressing mAbs in glycoengineered *P. pastoris*, antibody-mediated effector functions can be optimized by generating specific glycoforms [19]. Given the recent improvements in antibody and antibody fragment production in fungi [20], the humanized yeast expression system may compete in the near future with the established CHO-based process for the production of mAbs.

Identifying high-producing cell lines is critical for therapeutic protein development programs. Several high-throughput methods to quantify recombinant protein expression for specific *S. cerevisiae* [21] and *P. pastoris* clones have been previously described [22–24]. These methods consist of cultivation in 24-well plates or 96-well plates followed by purification and analysis. Unfortunately, high cell densities readily attained in bioreactors cannot be achieved in deep-well plates. Moreover, the pH and dissolved oxygen control present in bioreactors is absent in deep-well plates. As a result, the performance metrics of individual clones in large-scale bioreactors cannot be predicted based on high-throughput screening in microtiter plates.

In order to predict fermentation performance and simultaneously screen a large number of clones, several new technologies that miniaturize bioreactors have recently been reported. These include an 80-ml microfermenter battery [25], a 10-ml microbioreactor [26], a 10-ml scale bioreactor [27, 28], the 5-ml Applikon microreactor [29], the 3-ml Biocurve [30] and a 100-µl microbioreactor [31]. Unfortunately, many of these new technologies are either not commercially available or are unproven.

Here, we describe a novel, integrated high-throughout strain-screening and selection method that enabled us to isolate yeast cell lines that express mAbs at high titer and with uniform glycans. The combination of deep-well plate and small-scale bioreactor screening methods enabled us to routinely isolate glycoengineered *P. pastoris* clones that could be successfully scaled up to bioreactors. Once a promising strain was identified and verified in small-scale laboratory bioreactors (0.5 l), the strains were readily scaled-up to larger scale bioreactors [32].

Materials and methods

Cultivation in 96-well deep-well plates

Individual *P. pastoris* colonies transformed with the expression plasmid of interest and isolated from selective agar plates were transferred into individual wells of a 96-well deep-well titer block (USA Scientific, Ocala, FL) containing 600 µl per well BMGY comprising 100 m*M* potassium phosphate, 10 g/l yeast extract (BD, Franklin Lakes, NJ), 20 g/l peptone (BD, Franklin Lakes, NJ), 40 g/l glycerol, 18.2 g/l sorbitol, 13.4 g/l YNB (BD, Franklin Lakes, NJ), and 4 mg/l biotin. This "seed plate" was covered with a microporous rayon film and incubated for 48 h under our standard growth conditions: 24°C, 90% humidity, in a Multitron shaking incubator (ATR Biotech, Laurel, MD) at a speed of 840 rpm with a 3-mm throw. Multiple "expansion plates" were made by collecting 50 µl from the seed culture and inoculating this volume into 600 µl of fresh BMGY. Two to eight expansion plates were created for every seed plate depending upon the amount of material required for subsequent analysis. This two-step batch growth phase method allows the biomass to grow to saturation twice and therefore serves to normalize OD_{600} in microtiter plates. At the same time, a "master plate" was prepared by mixing 50 µl seed culture with 50 µl 40% glycerol in a 96-well microtiter plate. This was

covered with aluminum sealing film and stored at −80°C. Following 48 h under standard growth conditions, expansion plates were consolidated to one or two plates and induced with 600 µl fresh BMMY medium comprising 100 m*M* potassium phosphate, 10 g/l yeast extract (BD, Franklin Lakes, NJ), 20 g/l peptone (BD, Franklin Lakes, NJ), 20 ml/l methanol, 18.2 g/l sorbitol, 13.4 g/l YNB (BD, Franklin Lakes, NJ), and 4 mg/l biotin per well. Induction proceeded under standard growth conditions for 48 h without feeding. The supernatant was collected after centrifugation in a Beckman Allegra 6 centrifuge (Beckman Coulter, Fullerton, CA) at 2,500 rpm for 5 min.

Bead assay

About 50 µl supernatant from the 96-well plate cultures was diluted 1:1 with 50 m*M* Tris, pH 8.5, in a non-binding 96-well assay plate. For each 96-well plate, 2 ml of magnetic BioMag protein A suspension beads (Qiagen, Valencia, CA) were placed in a tube held in a magnetic rack. After 2–3 min when the beads had collected to the side of the tube, the buffer was decanted. The beads were washed three times with a volume of wash buffer equal to the original volume (100 m*M* Tris, 150 m*M* NaCl, pH 7.0) and resuspended in the same wash buffer. About 20 µl of beads were added to each well of the assay plate containing diluted samples. The plate was covered, vortexed gently and then incubated at room temperature for 1 h, vortexing every 15 min. Following incubation, the sample plate was placed on a magnetic plate inducing the beads to collect to one side of each well. On a Biomek NX Liquid Handler (Beckman Coulter, Fullerton, CA), the supernatant from the plate was removed to a waste container. The sample plate was then removed from the magnet and the beads were washed with 100 µl wash buffer. The plate was again placed on the magnet and the wash buffer was removed by aspiration. About 20 µl loading buffer (Invitrogen E-PAGE gel loading buffer containing 25 m*M* NEM (Pierce, Rockford, IL) was added to each well and the plate was vortexed briefly. Following centrifugation at 500 rpm on the Beckman Allegra 6 centrifuge, the samples were incubated at 99°C for 5 min and then run on an E-PAGE high-throughput precast gel (Invitrogen, Carlsbad, CA). Gels were covered with gel-staining solution (0.5 g Coomassie G250 brilliant blue, 40% MeOH, 7.5% acetic acid), heated in a microwave oven for 35 s, and then incubated at room temperature for 30 min. The gels were destained in distilled water overnight.

ELISA titer analysis

Monoclonal antibody titer was measured using a sandwich ELISA capturing secreted immunoglobulins with Pierce ImmunoPure Polyclonal goat anti-human kappa chain (Pierce, Rockford, IL). Each well of a Costar 96-well white polystyrene high-binding assay plate (Corning, Corning, NY) was coated with 0.1 µg Pierce ImmunoPure polyclonal goat anti-human kappa chain for 1 h at room temperature. The plate was washed on a Bio-Stack microtiter plate washer (BioTek Instruments, Winooski, VT) three times with 200 µl wash buffer (TBS, 0.2% Tween 20). Diluted sample (100 µl) was added followed by incubation for 1 h at room temperature. The plate was washed as described above and 100 µl per well of ImmunoPure rabbit anti-human IgG phosphatase (diluted 1:10,000; Pierce, Rockford, IL) was added. The plates were again incubated for 1 h at room temperature and washed. A solution of 4-MUP (100 µl; Virolabs, Chantilly, VA) was then added to each well and allowed to incubate for 30 min in the dark. Fluorescence was read on a Tecan Genios microplate reader at 350/465 nm (Tecan Systems, San Jose, CA).

ForteBio Octet titer analysis

Titer was also measured using ForteBio protein A biosensors in conjunction with the ForteBio Octet system (ForteBio, Menlo Park, CA). Titers were estimated by measurement of the binding rates of immunoglobulins in experimental samples and calculating the corresponding immunoglobulin concentration based on a standard curve. Protein A biosensors were presoaked at room temperature for 5 min in 200 µl "stripped" fermentation supernatant (supernatant from a previous experiment passed over a protein A column to remove all traces of antibody). About 100 µl of the supernatant from a 96-well culture plate was mixed with 100 µl sample diluent buffer (pH 7.4, 10 m*M* PBS, 0.02% Tween 20, 150 m*M* NaCl, 1 mg/ml BSA, 0.05% sodium azide). Alternatively, 20 µl of fermentation supernatant was mixed with 180 µl sample diluent buffer. The diluted sample was transferred to a 96-well black polystyrene nonbinding assay plate (Corning, Corning, NY). The sample plate and the sensor tray were allowed to incubate while shaking for an additional 5 min at 30°C prior to commencing sample measurement. The standard protein A experimental method was used for sample measurement and the data were analyzed using a curve fit (linear point-to-point). The standard curve was generated using commercially purified human polyclonal IgG antigen (Fisher Scientific, Hampton, NH).

PicoGreen assay

The DNA concentration in the supernatant was quantified using a previously reported method [33] with modification. Dilution buffer was prepared by adding 100 m*M* NaCl to 1× TE buffer and adjusting the pH to 8.8. Quant-iT

PicoGreen working buffer solution was then prepared by diluting Quant-iT PicoGreen reagent (Invitrogen, Carlsbad, CA) 1:200 in dilution buffer. Fermentation samples were incubated for one hour at 37°C with RNase A (Qiagen, Valencia, CA) at a final concentration of 50 μg/ml. Fermentation supernatant samples were then diluted 1:50 in the dilution buffer. About 100 μl of the sample mixture was then incubated with 100 μl Quant-iT PicoGreen working buffer for 4 min at room temperature in the dark. Fluorescence was read on a Tecan Genios microplate reader at 485/535 nm (Tecan Systems, San Jose, CA).

Release of N-linked glycans

N-glycans were released and separated from the glycoproteins using a previously reported method [16] with modification. The purified protein samples were reduced and carboxymethylated and the membranes were blocked as previously described. The samples were washed three times with water. The sample was deglycosylated by the addition of 30 μl 10 m*M* NH_4HCO_3 (pH 8.3) containing 1 mU N-glycanase (ProZyme, San Leandro, CA). After 16 h at 37°C, the solution containing the glycans was removed by centrifugation and evaporated to dryness.

MALDI time-of-flight mass spectrometry

Molecular weights of the glycans were determined using a Voyager DE linear MALDI time-of-flight (TOF) (Applied Biosystems) mass spectrometer with delayed extraction. The dried glycans from each sample well were dissolved in 15 μl of water, and then 0.5 μl of the glycan solution was spotted onto stainless-steel sample plates and mixed with 0.5 μl of S-DHB matrix (9 mg/ml dihydroxybenzoic acid/1 mg/ml 5-methoxysalicylic acid in 1:1 water/acetonitrile/0.1% trifluoroacetic acid) and allowed to dry. Ions were generated by irradiation with a pulsed nitrogen laser (337 nm) with a 4 ns pulse time. The instrument was operated in the delayed extraction mode with a 125 ns delay and an accelerating voltage of 20 kV. The grid voltage was 93.00%, guide wire voltage was 0.1%, the internal pressure was $<5\times10^{-7}$ torr (1 torr = 133 Pa), and the low mass gate was 875 Da. Spectra were generated from the sum of 100–200 laser pulses and acquired with a 500 MHz digitizer. $(Man)_5$-$(GlcNAc)_2$ oligosaccharide was used as an external molecular weight standard. All spectra were generated with the instrument in the positive-ion mode.

Fermentation screening

Fed-batch fermentations of glycoengineered *P. pastoris* were carried out in 0.5-l bioreactors (Sixfors multifermentation system; ATR Biotech, Laurel, MD) under the following conditions: pH 6.5, 24°C, 300 ml air-flow/min, and an initial stirrer speed of 550 rpm with an initial working volume of 350 ml (330 ml BMGY medium and 20 ml inoculum at a cell density of approximately 100 OD_{600}). Prior to inoculating the bioreactors, the cell density of the inoculums was normalized based on OD_{600} measurements in a standard spectrophotometer. IRIS multifermenter software (ATR Biotech, Laurel, MD) was used to increase the stirrer speed from 550 to 1,200 rpm linearly between hours 1 and 10 of the fermentation. Consequently, the dissolved oxygen concentration was allowed to fluctuate during the fermentation. The fermentation was executed in batch mode until the initial glycerol charge (40 g/l) was consumed (typically 18–24 h). A second batch phase was initiated by the addition of 17 ml of a glycerol feed solution to the bioreactor [50% w/w glycerol, 5 mg/l biotin and 12.5 ml/l PTM1 salts (65 g/l $FeSO_4\cdot7H_2O$, 20 g/l $ZnCl_2$, 9 g/l H_2SO_4, 6 g/l $CuSO_4\cdot5H_2O$, 5 g/l H_2SO_4, 3 g/l $MnSO_4\cdot7H_2O$, 500 mg/l $CoCl_2\cdot6H_2O$, 200 mg/l $NaMoO_4\cdot2H_2O$, 200 mg/l biotin, 80 mg/l NaI, 20 mg/l H_3BO_4)]. The fermentation was again operated in batch mode until the added glycerol was consumed (typically 6–8 h). The induction phase was initiated by feeding a methanol feed solution (100% w/w methanol, 5 mg/l biotin and 12.5 ml/l PTM1 salts) at 0.6 g/h. Methanol was typically fed for 36 h prior to harvest. The entire volume was removed from the reactor and centrifuged in a Sorvall Evolution RC centrifuge equipped with a SLC-6000 rotor (Thermo Scientific, Milford, MA) for 30 min at 8,500 rpm. The cell mass was discarded and the supernatant retained for purification and analysis.

Extended induction fermentation process

Identical to the fermentation screening process described above with the exception that the induction time was increased from 36 to 136 h [32].

Protein purification process

The mAb was purified using one-step affinity chromatography with streamline protein A (GE Healthcare, Piscataway, NJ). The resin was packed in a column and equilibrated with 50 m*M* Tris-HCl (pH 7). Fermentation supernatant was loaded into the column then washed using five column volumes of Tris-HCl, 1 *M* NaCl (pH 7) followed by five column volumes of the equilibration buffer. The mAb was eluted with five column volumes of a linear gradient from 50 m*M* Tris-HCl (pH 7) to 100 m*M* sodium citrate (pH 3). The elution fractions were neutralized by collection in a tube containing 1 *M* Tris-HCl (pH 7). The purifications were carried out at room temperature using an AKTAexplorer (GE Healthcare, Piscataway, NJ).

A SOURCE 30S resin (GE Healthcare, Piscataway, NJ) was used as a second purification step. The protein eluted from SL rProtein A was diluted fivefold with water to reduce conductivity to less than 10 mS/cm at pH 4.5 and loaded onto a SOURCE 30S column pre-equilibrated with 25 m*M* sodium acetate, pH 4.5. The column was washed with buffer A (12.5 m*M* sodium acetate, 12.5 m*M* sodium phosphate buffer, pH 6.0) and eluted with ten column volumes of buffer A with a linear gradient of 0–0.3 *M* NaCl.

Results

Strain generation

The construction of glycoengineered cell lines, expression vectors and transformation protocol has previously been described [15–18]. Briefly, empty glycoengineered cell lines were transformed by electroporation with an expression plasmid containing genes encoding antibody heavy and light chains under the control of the strong methanol-inducible AOX1 promoter. In our system the expression vector was stably integrated into the chromosome. After a single transformation event, hundreds of clones were isolated from positive selection plates (e.g. YPD agar plates containing the positive selection drug Zeocin). Additionally, the expression vector was transformed into multiple proprietary empty host cell lines in order to optimize expression and glycan quality. Consequently, for this project, thousands of clones were isolated after transformation and due to the high number of transformants, it was impossible to effectively screen these clones using a traditional shake flask expression format. Furthermore, several cell lines selected from the shake flask experiments based on titer results alone, performed poorly in bioreactors. This situation prompted us to develop a two-step screening process that employed an optimized 96-well deep-well plate expression protocol followed by small-scale fermentation screening.

Primary screening

Figure 1 summarizes our new integrated screening approach including analytical methods. Previously, we have described a 96-well deep-well plate protein expression assay designed for N-glycan screening [16]. This screening method was expanded to three-dimensional plate screening (titer, N-glycan profile and protein quality). Individual clones were transferred from agar plates into a single well of a 96-well deep-well plate. Figure 2a shows the layout of a typical plate after inoculation. Column 1 was intentionally left blank as this column served as a placeholder for titer standards added during analysis. Wells 2A through 2D and 7E through 7H represent positive control strains. These wells were inoculated with two previously characterized strains known to express mAbs in a predictable titer range. The remaining 80 wells were inoculated with uncharacterized clones.

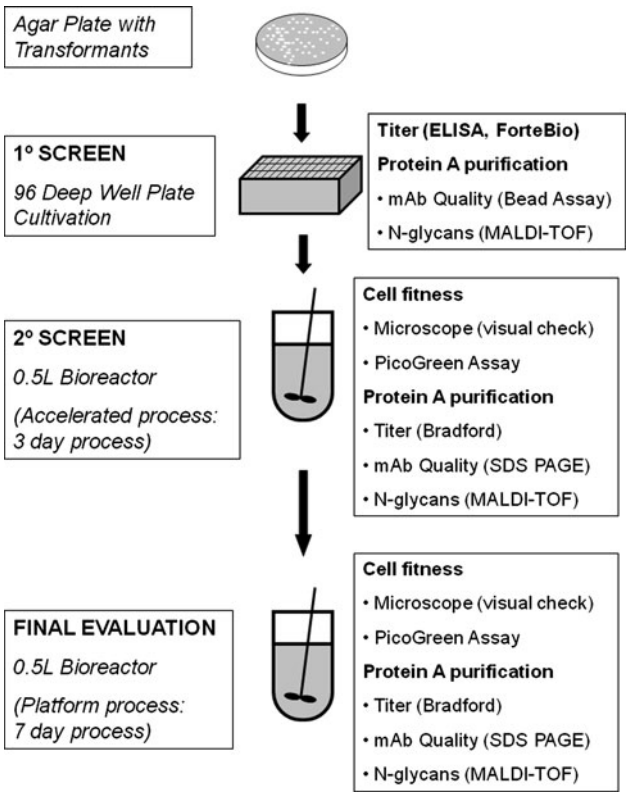

Fig. 1 Integrated screening workflow

Growth and induction of protein production were carried out as described in "Materials and methods". The resultant titer for each individual clone was determined using both ELISA and the ForteBio Octet system. The quality and uniformity of N-glycans was determined using mass spectrometry (MALDI-TOF, see "Materials and methods", data not shown). Protein quality was determined using a method referred to as the "Bead Assay" (see "Materials and methods" and Fig. 2b). This assay is a semiautomated small-scale recombinant protein A affinity capture step followed by analysis using SDS-PAGE under nonreducing conditions. Protein quality and titer were determined by visual evaluation of SDS-PAGE gels. Gel imaging software (e.g. ImageJ, NIH, Bethesda, MD) was also used to calculate titer based on the titer standards (data not shown). From a single 96-well plate cultivation step and subsequent data analysis, promising strains (typically 1–5% of the total population) were selected based on three performance criteria, namely titer, N-glycan uniformity and protein quality.

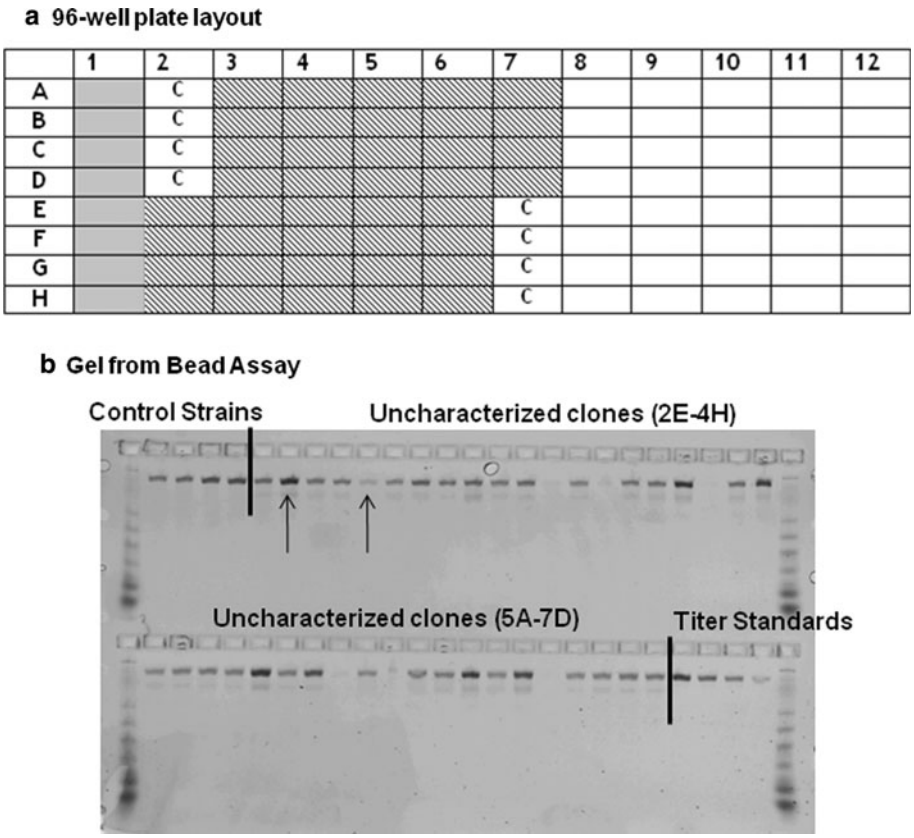

Fig. 2 Sample of results generated during primary screening. **a** Layout of a typical 96-well deep-well plate. Column 1 is intentionally left blank as this column serves as a placeholder for titer standards added during analysis. Wells 2A through 2D and 7E through 7H represent positive control strains (two previously characterized strains that express mAbs at known titer). The remaining 80 wells are inoculated with uncharacterized clones. **b** Example of SDS-PAGE gel obtained from the bead assay analysis (see "Materials and methods"). The arrows denote two clones that display a significant difference in titer

Secondary screening

From our experience, we know that the primary screening step does not necessarily identify strains compatible with our platform fermentation process. This is attributed to different growth and induction conditions in a bioreactor versus a deep-well plate. Some strains yielding high titer and N-glycan quality in deep-well plates fail in the bioreactor due to excessive cell lysis. Therefore, an additional screening step was required, namely to scale-up expression from 96-well plates to small-scale bioreactors, a process dubbed "fermentation screening". We utilized 0.5-l bioreactors and designed an accelerated 3-day fermentation protocol that successfully predicted the performance observed in our platform fermentation process. The new fermentation screening protocol enabled us to execute two fermentations per bioreactor per week. In comparison, the platform fermentation process typically requires 7 days, thus limiting throughput to one fermentation per bioreactor per week. The accelerated fermentation screening protocol is characterized by reduced glycerol batch time and shorter induction time. The glycerol batch time was reduced by increasing the cell density of the inoculum to about 100 OD_{600}. The methanol induction phase was reduced from 136 to 36 h. Despite the reduction in induction time, the performance differences between individual strains were easily identified. Samples generated from the 0.5-l bioreactor were purified and analyzed for titer, N-glycans, protein quality and extent of cell lysis (see "Materials and methods").

The analytical data collected from secondary screening allowed us to evaluate multiple performance criteria for each clone, namely titer, N-glycan uniformity, protein quality and cell fitness. Cell fitness or robustness was assessed by evaluating the cells under a microscope at the end of the fermentation. In addition, the DNA concentration in the supernatant was measured to quantify the extent of cell lysis. Typically strains classified as displaying a high degree of cell lysis showed extensive cell debris under the microscope and yielded a supernatant DNA concentration greater than 5 μg/ml. Yeast strains classified as showing a light degree of cell lysis displayed low levels of cell debris under the microscope and yielded a supernatant DNA concentration between 1 and 5 μg/ml. To illustrate this method in practice, Fig. 3 shows a representative example of antibody with high and low quality N-glycans. In terms of the degree of cell lysis, a low degree of cell lysis refers to a clone with less than 1 μg/ml in the fermentation supernatant at the conclusion of a 130-h bioreactor cultivation. A light degree of lysis corresponds to DNA concentrations between 1 and 5 μg/ml at the end of the cultivation. A high degrees of lysis corresponds to DNA concentrations exceeding 5 μg/ml.

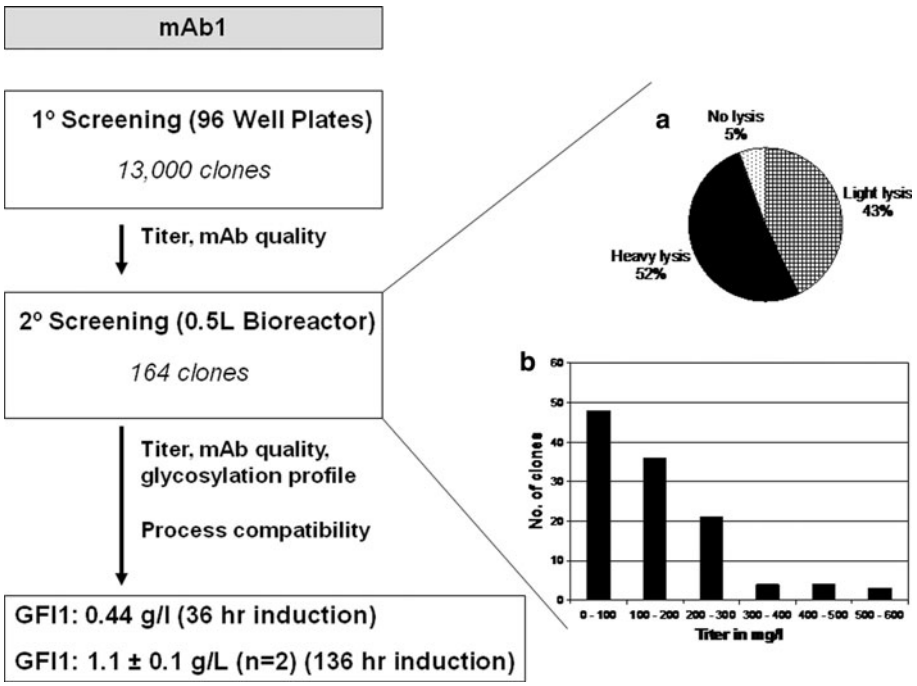

Fig. 3 Results for mAb1 (secondary screening and final evaluation step). **a** 164 glycoengineered *P. pastoris* clones expressing mAb1 were tested in 0.5-l bioreactors (secondary screening). Of the 164 clones, only 5% showed no degree of cell lysis while 43% showed a light degree of cell lysis (acceptable). However, 52% showed excessive cell lysis (unacceptable). **b** The 164 clones were distributed among five final fermentation titer distribution categories and shown as a bar graph. All titers are the titers after protein A purification, normalized to the bioreactor working volume. Only four clones yielded a titer greater than 500 mg/l in the fermentation screening protocol. The titer of 48 clones was not measured as these fermentations were prematurely terminated due to excessive foaming associated with excessive cell lysis

Example

To illustrate the new workflow, we have summarized our results for two mAbs named mAb1 and mAb2. During the execution of the project, 13,000 mAb1-expressing clones were generated by transforming 42 empty host cell lines with the expression vector in 119 transformations. In a similar fashion, 4,000 mAb2-expressing clones were generated from 27 empty host cell lines. All of these 13,000 mAb1-expressing clones and 4,000 mAb2-expressing clones were subjected to the primary screening process. At the end of primary screening, 164 promising mAb1-expressing clones and 226 mAb2-expressing clones were identified for further evaluation in secondary screening (fermentation screening).

Figure 3a shows that 52% of the 164 mAb1-expressing clones tested in the secondary screening step failed our fermentation fitness test by showing a significant degree of cell lysis (determined by evaluating the cells under a standard optical microscope and by quantifying the DNA concentration in the fermentation supernatant). Figure 3b shows the titer distribution of the 164 mAb1-expressing clones tested. Note that the most category with the greatest number of clones was 0–200 mg/l (47 clones). Conversely the category with the fewest clones was the highest titer category of 500–600 mg/l (4 clones). Note that the sum of all clones in Fig. 3b is only 116 and not 164 clones. The final titer for 48 clones was not determined because these fermentation experiments were terminated early due to excessive foaming associated with elevated cell lysis. However, these 48 clones were included in Fig. 3a under the category of "heavy lysis".

Figure 4a shows that 23% of the 226 mAb2-expressing clones tested in the secondary screening step failed our fermentation fitness test by showing a significant degree of cell lysis in fermentation. Figure 4b shows the titer distribution of the 226 clones tested. Note that the category with the greatest number of clones was again 0–50 mg/l (85 clones). Conversely, the category with the fewest clones was the highest titer category of 150–200 mg/l (12 clones). Note that the sum of all clones in Fig. 4b is only 205 and not 226 clones. The final titer for 21 clones was not determined because these fermentation experiments were terminated early due to excessive foaming associated with elevated cell lysis. However, these 21 clones were included in Fig. 3a under the category of "heavy lysis".

Together, Figs. 3 and 4 show that most clones were unsuitable for mAb production because of low titer

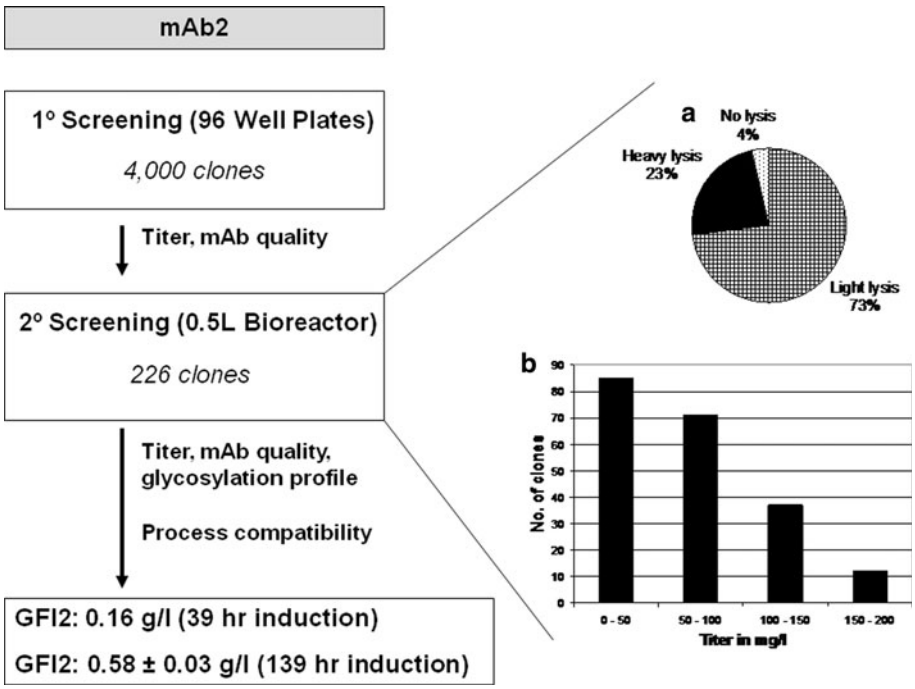

Fig. 4 Results for mAb2 (secondary screening and final evaluation step). **a** 57 glycoengineered *P. pastoris* clones mAb2 were tested in 0.5-l bioreactors (secondary screening). Of the 57 clones, only 12% showed no cell lysis while 49% showed a light degree of cell lysis (acceptable). However, 39% showed excessive cell lysis (unacceptable). **b** The 57 clones were distributed among five final fermentation titer distribution categories and shown as a bar graph. All titers are the titers after protein A purification, normalized to the bioreactor working volume. Only six clones yielded a titer greater than 150 mg/l in the fermentation screening protocol. The titer of six clones was not measured as these fermentations were prematurely terminated due to excessive foaming associated with excessive cell lysis

and/or low cell viability in fermentation. In addition to screening for titer and cell viability, we also evaluated N-glycan uniformity and protein quality to further eliminate unsuitable clones (data not shown). From the primary and secondary screening process, clone GF1 was identified as a promising candidate strain expressing mAb1 and cultivated in the platform fermentation process [32].

Figure 5 summarizes key performance data generated in the platform fermentation process and secondary screening process, both generated in 0.5-l bioreactors. Strain GF1 yielded a titer of 1.1±0.1 g/l (n=2) in the platform fermentation process (136 h induction) and yielded very high N-glycan uniformity and high protein product quality by SDS-PAGE. The same strain yielded a titer of 0.44 g/l during secondary screening, also with very high N-glycan and protein quality. Similarly, strain GF2 yielded an average titer of 0.58±0.03 g/l (n=2) in the platform fermentation process (136 h induction) and yielded very high N-glycan uniformity and high protein product quality by SDS-PAGE. To further illustrate the reproducibility of our process, we have also recently reported the titers for strain GF1 (also known as YGLY4140) in bioreactors of volume 0.5, 3, 15, and 40 l [32]. An average titer of 1.26±0.05 g/l was reported from a 146-h cultivation process.

Discussion

Historically, our strategy was to cultivate yeast transformants in simple shake flasks to confirm antibody expression. Yeast clones positive for recombinant protein expression were then directly cultivated in bioreactors. This strategy proved inefficient for us as many yeast clones ultimately yielded low antibody titer and/or quality (data now shown). Therefore, to improve our success rate for identifying clones with the desired antibody product profile and create a more efficient process, the goal of the studies described here was to develop an improved screening process.

In our specific workflow, highly engineered yeast strains [5, 15–19] are transformed with a vector containing antibody expression genes and the zeocin resistance transformation marker. This yeast transformation process is believed to generate significant phenotypic diversity. We acknowledge that the exact mechanisms responsible for this diversity are not understood but we can speculate on potential causes. Specifically, integration copy number is not specifically controlled, although it is known that gene dosage can influence productivity and titer [34–36]. Moreover, zeocin is known to be a mutagen [37, 38]. Currently, we are conducting studies to further elucidate the source of phenotypic diversity.

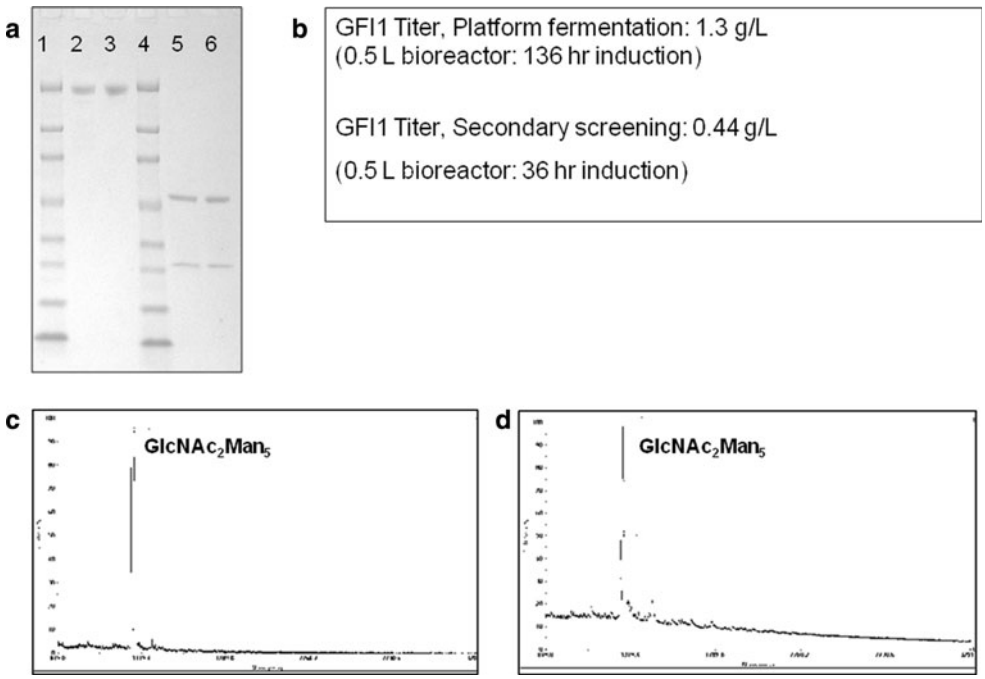

Fig. 5 Key performance metrics of strain GF1 expressing mAb1 in the extended induction fermentation process (0.5-l bioreactors) showing high N-glycan uniformity and high protein product quality. All titers are titers after protein A purification, normalized to the bioreactor working volume. **a** SDS-PAGE gel. *Lane 2*: Nonreducing extended induction fermentation process, 0.5-l bioreactor, 136 h induction. *Lane 3*: Nonreducing fermentation screening protocol, 36 h induction. *Lane 5*: Reducing extended induction fermentation process, 0.5-l bioreactor, 136 h induction. *Lane 6*: Nonreducing fermentation screening protocol, 36 h induction. **b** Titer summary. **c** N-glycan profile determined by MALDI-TOF mass spectrometry for the extended induction fermentation process, 0.5-l bioreactor, 136 h induction. **d** N-glycan profile MALDI Time-of-Flight (TOF) mass spectrometry determined for the fermentation screening protocol, 0.5 l bioreactor, 36 h induction

As mentioned above, the goal of the studies reported here was to design an efficient method for identifying clones able to produce therapeutic mAbs at manufacturing scale. To this end we have developed and implemented an integrated high- and medium-throughput screening workflow to identify glycoengineered yeast clones that express mAbs using standard microbial fermentation equipment. This two-step process combines the high-throughput advantages of 96-well plates with a novel fermentation scale-down protocol in 0.5-l bioreactors. This process overcomes the limitations of standard screening methods that typically utilize shake flasks to evaluate a limited number of transformants under conditions that often do not reflect scale-up performance in bioreactors. Cell densities are typically lower in shake flasks relative to bioreactors. Moreover, process controls including carbon feed, pH, and dissolved oxygen control, which are difficult to manipulate in shake flasks, are readily controlled in a bioreactor.

Our screening method employing 96 deep-well plates and small-scale bioreactors, has been utilized to identify robust, production cell lines producing high titer in industrial scale processes [32]. In fact, yeast strain YGLY4140, described by Potgieter et al. [32] was identified during this study. Potgieter et al. presented a detailed characterization, demonstrating similar antibody productivities across a broad range of bioreactor scales (0.5, 3, 15 and 40 l) using strain YGLY4140. They also showed that the antibody produced in different bioreactors showed similar characteristics by SDS-PAGE analysis, SEC-HPLC and Biacore antigen binding assays.

In summary, following a standard yeast DNA transformation, we were able to consistently identify robust clones that express mAbs at high titer, with uniform N-glycans in bioreactors. The process typically requires 6–8 weeks, which includes small-scale purification and analytical characterization. This approach allows the production of sufficient material for preclinical development using very short, aggressive project time-lines, thereby greatly reducing the time and cost required for drug development.

Although our screening platform is currently tailored specifically for the identification of antibody-producing cell lines, it can be readily adapted for the selection of cell lines expressing other classes of therapeutic glycoproteins (results not shown). Additionally, this integrated process is currently used in our laboratory to support strain engineering efforts and media optimization studies (results not shown). The presented workflow can be readily automated utilizing standard colony-picking and liquid-handling

robots to further increase the throughput and reduce intraassay variation.

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