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REVIEW

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Reexamining opportunities for therapeutic protein production in eukaryotic microorganisms

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Abstract

Antibodies are an important class of therapeutics and are predominantly produced in Chinese Hamster Ovary (CHO) cell lines. While this manufacturing platform is sufficiently productive to supply patient populations of currently approved therapies, it is unclear whether or not the current CHO platform can address two significant areas of need: affordable access to biologics for patients around the globe and production of unprecedented quantities needed for very large populations of patients. Novel approaches to recombinant protein production for therapeutic biologic products may be needed, and might be enabled by non-mammalian expression systems and recent advances in bioengineering. Eukaryotic microorganisms such as fungi, microalgae, and protozoa offer the potential to produce high-quality antibodies in large quantities. In this review, we lay out the current understanding of a wide range of species and evaluate based on theoretical considerations which are best poised to deliver a step change in cost of manufacturing and volumetric productivity within the next decade.
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antibody, CHO, expression system, glycoengineering, process design, therapeutic protein production

1 | INTRODUCTION

Biologic drugs continue to drive growth in the pharmaceutical industry, with sales reaching $201B in 2013 and biologics comprising 26% of all new drug approvals in the United States between 2010 and 2014 (Highsmith, 2015; Walsh, 2014). Monoclonal antibodies (mAbs) comprise the dominant class, making up 38.6% of 2013 sales. Product development shows no signs of stopping: 183 biotech products were in Phase III clinical trials in 2014 (Tufts Center for the Study of Drug Development, Outlook 2015). As patent rights expire for many monoclonal antibody products and biosimilar products are developed at lower prices, patients in new geographies should gain access to these drugs and sales are expected to rise (Ecker, Jones, & Levine, 2015).

Mammalian cell lines, in particular Chinese Hamster Ovary (CHO) cells, are the expression system of choice for biologic drugs and continue to gain prominence in commercial manufacturing processes. The development of new mAbs and other proteins with complex post-translational modifications has, in part, propelled this selection (Walsh, 2014), along with efforts to streamline process development. An estimated, 8.5 metric tons of drug substance were manufactured in CHO cells in 2010, with demand expected to rise to 13.4 metric tons in 2016 (Walsh, 2014).

Production processes in CHO have become well-developed. Typically, they comprise cultivation in stainless steel bioreactors, harvest by centrifugation, purification by a series of chromatographic separations, virus inactivation and removal steps, and fill and finish.

The adoption of fed-batch processes with optimized media has driven production titers to typical commercial values of 1–5 g/L today and as high as 10–13 g/L in a 14-day production run, with the potential of reducing the cost of manufacturing from $300/g to as low as $50/g of drug product (Kelley, 2009). In its current form, a CHO-based manufacturing platform appears sufficient to supply current markets concentrated in North America and Western Europe (Kelley, 2009). Nonetheless, it is unclear whether or not this model can attain lower costs and higher productivity levels needed to reach patient populations currently not supported by biologics.

Patients in the developing world are heavily affected by diseases such as HIV and cancer. To make biologic medicines available in these areas, the costs of manufacturing need to be substantially reduced. The Gates Foundation has advised that for routine treatment of HIV or infectious diseases in the developing world, the cost of monoclonal antibodies must drop to $10/g or less (Hadley, 2013). While it is common practice for biopharmaceutical companies to sell vaccines at lower prices in the developing world, it would be economically unsustainable for them to sell large volumes of antibodies at a price substantially lower than the current cost of production. The cost of biologics manufacturing is driven by two main factors: the fixed cost of running a facility, including depreciation, and the variable cost per batch, including media, consumables, and utilities. Mammalian cells grow slowly, require sophisticated media formulations, and rely on expensive separation methods such as chromatography. Increasing rates of facility utilization across the industry have reduced the burden of fixed costs, but reductions in variable costs will also be required to achieve costs significantly below $50/g.

The application of biologic drugs for prevalent chronic diseases, such as heart disease and Alzheimer's disease, represents a potentially important opportunity to improve the breadth of patient care. The size of these affected populations, however, represents a potential future manufacturing challenge. For example, if a monthly dose of 1 g of antibody is prescribed to a patient population of one million, the required 12 metric tons would almost double the expected production in CHO in 2016. These one million patients would comprise less than 20% of the patient population affected by Alzheimer's disease in 2014 in the United States alone (Alzheimer's Association, 2014). Based on current production processes, building new manufacturing sites to meet this capacity will require enormous capital investments.

To address these considerations, a new approach to recombinant protein production for therapeutic biologic products may be needed. Looking to other industries for inspiration, the production of industrial enzymes has many similarities to the production of therapeutic proteins, but is dominated by processes that generate metric tons of material per batch at significantly lower cost (Singhania, Patel, & Pandey, 2010), as low as $2–10 per kilogram (Ellilä et al., 2017). These processes are built around microbial host organisms which grow quickly and robustly in fermenters up to 300,000 L in volume, with titers as high as 100 g/L for some enzymes (Cherry & Fidantsef, 2003). Processes for purification are more simplistic to meet less stringent product purity requirements, incorporating precipitation and crystallization where feasible to replace chromatography steps. Similarly, the plasma fractionation industry can use simple, low-cost purification processes for protein therapeutics because the quality of the plasma feedstock is tightly controlled (Bertolini & Hayes, 2013). The application of these approaches to manufacturing parenteral biologic drugs is not straightforward, but these examples suggest the selection of the host is an important initial element in minimizing production costs.

Even with processes for production and purification in place, alternative hosts will require substantial genetic engineering to satisfy requirements for product quality. Advances in molecular biology and bioengineering have placed researchers in a position to address these concerns. It is now feasible to quickly and efficiently engineer simpler host organisms (Hutchison et al., 2016) and establish new platforms for production of therapeutic glycosylated proteins (Wildt & Gerngross, 2005). Faster, cheaper sequencing has extended biological understanding of a wide range of organisms. New gene editing tools enable multiplexed strain development and approaches for targeted engineering (Carr & Church, 2009). Drug developers are learning how antibody sequences and glycoforms can be modified to increase drug efficacy (Presta, 2008). Engineering of desired glycoforms has been demonstrated in both mammalian and fungal cells (Beck et al., 2008). These tools together create the potential to develop expression systems that enable step changes in cost of manufacturing and volumetric productivity.

2 | TYPES OF ORGANISM CONSIDERED

Although many classes of organisms have been engineered to produce recombinant proteins, this review focuses on organisms that appear best poised to meet needs for the production of monoclonal antibodies within the next decade. Drugs manufactured in other mammalian cell lines, insect cell lines, transgenic animals, and plants have been approved by regulators, but grow too slowly to enable a step-change in total productivity that is required for very high-volume products. While there are advances to enable bacteria to properly fold or glycosylate complex proteins (Merritt, Ollis, Fisher, & Delisa, 2013), engineering of these functions is in our opinion not far enough along to reach target scales in the near term. Eukaryotic microorganisms are an ideal intermediate, growing quickly like bacteria, but already containing the organelles and functions required for complex protein processing and secretion.

In this review, we lay out the current state of understanding of the suitability of fungi, microalgae, and protozoa for production of therapeutic proteins. We have identified species from each category with sequenced genomes and proven expression of complex heterologous proteins. *Saccharomyces cerevisiae* is a well-studied reference organism that has been widely used for protein expression. Methylotrophic yeasts *Pichia pastoris* (reclassified as *Komagataella phaffii* and *Komagataella pastoris*) (Cereghino & Cregg, 2000), *Hansenula polymorpha* (reclassified as *Ogataea polymorpha* and *Ogataea parapolymorpha*) (Stöckmann et al., 2009), and *Kluyveromyces lactis* (van Ooyen et al., 2006) were developed as expression systems

with inducible promoters activated by a change in carbon source. Filamentous fungi *Trichoderma reesei* (anamorph of *Hypocrea jecorina*) (Singh et al., 2015) and *Aspergillus oryzae* (Fleißner & Dersch, 2010) are heavily used for homologous and heterologous enzyme production. Microalgae *Chlamydomonas reinhardtii* (Specht, Miyake-Stoner, & Mayfield, 2010) and diatom *Phaeodactylum tricornutum* (Hempel, Lau, Klingl, & Maier, 2011) have been considered for applications in both biofuel and recombinant protein production. Protozoans *Tetrahymena thermophila* (Jayaram et al., 2010) and *Leishmania tarentolae* (Basile & Peticca, 2009) are both commercially available as expression systems.

We compare here potential expression systems across characteristics relevant for manufacturing including toolkits for strain engineering, requirements for glycoengineering, flexibility of bioprocess options, and secretion capacity. While to date, there has been no consensus on the best non-mammalian expression system, we outline key trade-offs and identify areas where more research and development is needed to make comparisons.

3 | STRAIN ENGINEERING

Methods to manipulate genomes are a basic requirement for any host organism: the gene for the recombinant protein must be incorporated into the genome for it to be transcribed. For large-scale production, further genetic engineering offers a powerful tool to increase productivity and improve product characteristics.

Transformation methods for foreign genes are not fundamentally different between CHO and alternative expression systems. Incorporating single genes into mammalian and microbial hosts has relied on well-established methods such as electroporation, microparticle bombardment, PEG- or lithium-mediated transformation, and *Agrobacterium*-mediated transformation (Table 1). Transformation efficiencies vary widely across organisms and methods. Efficiencies are highest for yeast, at 10^5–10^7 transformants per microgram of plasmid DNA (Kawai, Hashimoto, & Murata, 2010). Transformation into filamentous fungi has been more challenging because of the fungal cell wall—reported efficiencies are typically on the order of 100–1,000 transformants per microgram of DNA (Fleißner & Dersch, 2010). Agrobacterium-mediated methods has been shown to generate high numbers of transformants in *Aspergillus* (de Groot et al., 1998) and in *C. reinhardtii* (Kumar, Misquitta, Reddy, Rao, & Rajam, 2004), the latter of which produced only 10–20 transformants per microgram of DNA using particle bombardment (Boynton et al., 1988). Electroporation was recently demonstrated in *P. tricornutum* at 1,000 transformants per µg of DNA (Miyahara, Aoi, Inoue-Kashino, Kashino, & Ifuku, 2013), a significant improvement over the 10 per microgram generated through particle bombardment (Apt, Kroth-Pancic, & Grossman, 1996). Successful transformations have been demonstrated for *T. thermophila* and *L. tarentolae* but efficiencies were not reported. Across organisms, application of higher efficiency methods and improvement of existing protocols has led to increases in transformation efficiencies that make foreign gene integration and genome-level engineering more feasible.

Targeting a gene to a defined locus is more straightforward in alternative hosts than in CHO. Non-homologous random integration predominates in CHO, in contrast to the homologous recombination naturally favored by *S. cerevisiae* (Zheng & Wilson, 1990), *L. tarentolae* (Papadopoulou & Dumas, 1997), and *T. thermophila* (Cassidy-Hanley et al., 1997). Other yeasts and filamentous fungi naturally display a preference for non-homologous end-joining (NHEJ), but strategies have been developed to increase homologous recombination rates up to 100%: the use of longer homology sequences to flank the integration cassette, or deletion of NHEJ-promoting proteins (Kück & Hoff, 2010; Wagner & Alper, 2016). In most species of microalgae, foreign genes integrate homologously into chloroplasts but randomly into nuclei (Gong, Hu, Gao, Xu, & Gao, 2011).

Recently developed gene editing techniques are poised to revolutionize gene editing across organisms. For example, CRISPR-Cas9 has already been successfully demonstrated in all classes of organisms represented in Table 1 (Jiang, Brueggeman, Horken, Plucinak, & Weeks, 2014; Katayama & Tanaka, 2016; Liu, Chen, Jiang, Zhou, & Zou, 2015; Nymark, Sharma, Sparstad, Bones, & Winge, 2016; Ronda et al., 2014; Sollelis et al., 2015; Weninger, Hatzl, Schmid, Vogl, & Glieder, 2016). There are, however, some barriers to widespread adoption in the near term and additional investment will be required to further develop these techniques. Off-target binding of the Cas9/single-guide RNA complex has been extensively studied, but it can be difficult to predict genome cutting and mutation with confidence (Wu, Kriz, & Sharp, 2014). The heritability of mutations introduced using CRISPR has primarily been studied in plants, where the second generation has been demonstrated to inherit anywhere from 20% to 96% of the original mutations (Mao, Botella, & Zhu, 2016). A deeper understanding of reversion potential and genomic stability for alternative hosts would be needed before adoption for commercial production.

Development of heterologous protein-producing strains can proceed much faster in eukaryotic microorganisms than in CHO because of targeted gene integration and faster growth. Homologous recombination, available in eukaryotic microbial hosts, simplifies the engineering of gene knock-ins and reduces the number of clones that must be screened. Growth time is a limiting factor when screening mammalian clones, both at small scale and in bioreactors, because the typical doubling time of CHO is 14–36 hr. In contrast, the doubling times of yeast, fungi, and *T. thermophila* are 1.5–4 hr, while microalgae and *L. tarentolae* are 5–15 hr (Fernández & Vega, 2013). Taken together, generation of a master cell line using a eukaryotic microorganism can be completed in approximately 1 month compared to a typical 6 months for CHO, allowing much earlier clinical evaluation of new molecules.

In addition to being faster, strain engineering is also more straightforward with simpler organisms. Chinese hamsters became popular for tissue culture studies in the 1940's because they have fewer chromosomes than mice or rats (Jayapal, Wlaschin, Hu, & Yap, 2007). Extending that idea to its extreme, synthetic biology researchers have designed a bacterial genome that includes only the minimum set of genes required for robust growth

TABLE 1 Genome characteristics and traditional DNA integration methods for alternative hosts.

				Method of DNA integration			
Class	Species	Genome size (Mbp)	# of chromosomes	Electroporation	Particle bombardment	PEG- or lithium-mediated	Bacterially mediated
Mammalian	Chinese hamster ovary (CHO)	2,450	21	x	x	x	
Yeast	*Saccharomyces cerevisiae*	12	16	x	x	x	
Yeast	*Pichia pastoris*	9.4	4	x		x	
Yeast	*Hansenula polymorpha*	9.5	6	x			
Yeast	*Kluyveromyces lactis*	11	6	x		x	
Filamentous fungi	*Trichoderma reesei*	34	7	x	x	x	x
Filamentous fungi	*Aspergillus oryzae*	37	8	x	x		x
Microalgae	*Chlamydomonas reinhardtii*	120	17	x	x		x
Microalgae	*Phaeodactylum tricornutum*	27	33	x	x		x
Protozoa	*Tetrahymena thermophila*	104	225	x	x		
Protozoa	*Leishmania tarentolae*	30	36	x			

All of these species have been sequenced and reference genomes are available through the NCBI Handbook (http://www.ncbi.nlm.nih.gov/genome/browse/). Electroporation, particle bombardment, chemically mediated methods, and bacterially mediated methods have all been used for foreign DNA integration (Apt et al., 1996; Basile & Peticca, 2009; Cassidy-Hanley et al., 1997; Cereghino & Cregg, 2000; Fleißner & Dersch, 2010; Karas et al., 2015; Karrer, 2000; Miyahara et al., 2013; Penttilä, Nevalainen, Rättö, Salminen, & Knowles, 1987; Potvin & Zhang, 2010; Schaffrath & Breunig, 2000; Singh et al., 2015; Specht et al., 2010; Van Dijk et al., 2000; Wurm, 2004).

(Hutchison et al., 2016). In parallel, other researchers have developed cell-free systems for protein synthesis made up of highly concentrated cellular extracts typically from *Escherichia coli* (Casteleijn, Urtti, & Sarkhel, 2013). These cell-free systems are currently limited in many of the same ways as bacteria with respect to complex post-translational modifications like glycosylation, but remind us that a small genome provides a simple chassis for strain engineering.

For an organism with a small genome, it becomes feasible to rewrite large portions of the genome and sequence the final strain at low cost (Carr & Church, 2009). Sequencing alignment is more straightforward, so analysis can be completed more quickly and mutations can be more confidently identified. The cost of sequencing the methylotrophic yeast *P. pastoris* with 100× coverage is on the order of only $100 per sample, compared to tens of thousands of dollars for CHO. At this low cost, strategies for strain improvement using forward and reverse genetics become available and scalable to high-throughput approaches.

The genomes of all of the eukaryotic microorganisms compared here are one to two orders of magnitude smaller than that of CHO (Table 1). Methylotrophic yeast have the smallest genomes at approximately 10 Mbp, and also benefit from close homology to the well-annotated reference organism *S. cerevisiae*. The genome of *S. cerevisiae* is slightly larger, at 14 Mbp. The diatom *P. tricornutum*, the protozoan *L. tarentolae*, and the filamentous fungi have genomes of 25–35 Mbp, still one hundred times smaller than that of CHO. The genomes of *C. reinhardtii* and *T. thermophila* are ten times larger than those of the yeast, which could complicate strain engineering.

Genome organization is also important for host organisms because the number of chromosomes affects cell line stability, which must be well-characterized for regulatory approval. CHO cells are notoriously heterogeneous populations, with even the number of chromosomes varying within the low twenties (Deaven & Petersen, 1973). Organisms with fewer chromosomes typically have less repetitive genomes, and thus are much less likely to survive after losing part or all of a chromosome, increasing overall clonal stability. The *T. thermophila* genome is not only large, but is also organized into 225 chromosomes in two nuclei—a staggering level of complexity that likely precludes routine sequencing. While the genomes of *P. tricornutum* and *L. tarentolae* are not as large, their higher chromosome counts (Table 1) could complicate strain engineering. Further research into cell line stability is required to validate these hypotheses and allow direct comparison across these classes of organisms.

The eukaryotic microorganisms shown in Table 1 all have established methods for genetic transformation and offer advantages in faster doubling times and simpler genomes that could dramatically accelerate strain engineering. Further work is required to improve functional annotations of the genomes of these organisms, to directly compare transformation efficiencies, and to study genomic stability under a variety of conditions.

4 | GLYCOSYLATION

N-linked glycosylation is one of the most common post-translational modifications and plays an essential role in the effector function and pharmacokinetics of antibodies and other therapeutic proteins. Human-like N-glycan structures are complex, containing five different sugars in a multi-antennary structure (Figure 1a): mannose (Man), N-acetylglucosamine (GlcNAc), fucose (Fuc), galactose (Gal), and sialic acid. Glycosylation is a complex process that occurs in the endoplasmic reticulum and Golgi apparatus as a protein is secreted, as described in detail in recent reviews (Beck et al., 2008; Hossler, Khattak, & Li, 2009).

CHO cells have a proven track record of producing human-like N-linked glycoforms that are compatible and bioactive in humans (Jayapal et al., 2007). These glycoforms are heterogeneous, with different numbers of branches and varying extents of fucosylation, galactosylation, and sialic acid capping. Significant effort has gone into characterizing the impact of process conditions on glycosylation profiles (Hossler et al., 2009). A small number of potentially immunogenic glycoforms can be generated by CHO (Jefferis, 2009), but this effect is minimized through glycan screening during cell line development.

The eukaryotic microorganisms under consideration here naturally contain the necessary organelles (e.g., ER and Golgi) and some of the enzymes (e.g., mannosyl transferases) required to naturally produce complex glycoforms (Figure 1a). *L. tarentolae* has glycan processing pathways that produce glycans closest to the human form, with attached fucose and galactose residues. The diatom *P. tricornutum* can also produce fucosylated glycoforms. Filamentous fungi *T. reesei* and *A oryzae* and protozoan *T. thermophila* produce smaller glycoforms that match intermediates in the human glycosylation pathway, but contain only mannose and N-acetylglucosamine. In yeast, mannose chains are elongated in the Golgi apparatus to form immunogenic structures, but the details differ by species. *S. cerevisiae* and *K. lactis* naturally generate glycoforms with over 30 mannoses, while hyperglycosylation is much less elaborate in *P. pastoris* and *H. polymorpha* (De Pourcq et al., 2010). The glycoforms produced by *C. reinhardtii*

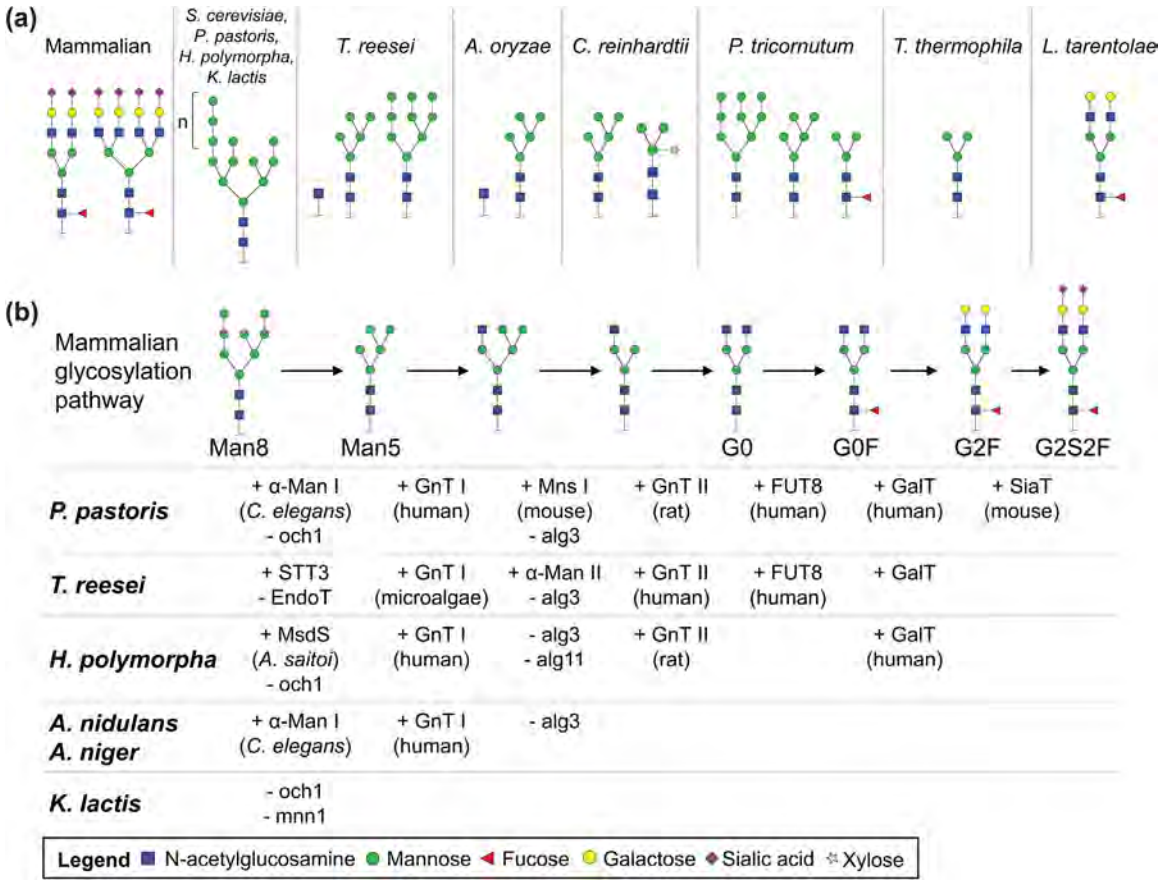

FIGURE 1 Glycan structures and glycoengineering of host organisms. (a) Native glycoforms that have been detected experimentally in mammalian cells (Hossler et al., 2009), yeast (Anyaogu & Mortensen, 2015), filamentous fungi (Maras, Van Die, Contreras, & Van Den Hondel, 1999; Stals et al., 2004), microalgae (Baïet et al., 2011; Mathieu-Rivet et al., 2013; Vanier et al., 2015), and protozoa (Basile & Peticca, 2009; Weide et al., 2006). Structures shown do not encompass all possible forms but demonstrate relevant enzymatic reactions such as mannose trimming and range of sugar monomers. Multiple glycan structures were included when data were available about heterogeneity. (b) Demonstrated glycoengineering in different species. Both the glycans that have been created and the enzymes used are documented. Symbolic nomenclature follows the guidelines established by the Nomenclature Committee of the Consortium for Functional Glycomics. Images were created using GlycanBuilder (Ceroni, Dell, & Haslam, 2007; Damerell et al., 2012)

include xylose, which is common to plant cells and is immunogenic. The glycoforms produced by some of these organisms may be acceptable for certain therapeutic proteins, but production of antibodies will likely require modifications to the glycan structures.

Enzymatic glycosylation is one strategy that has been proposed to produce therapeutic proteins with homogeneous human-like glycoforms (Wang & Amin, 2014). In this chemical approach, reactions are performed between purified protein and separately produced glycan structures. An EndoS enzyme is used to cleave the existing glycan between the two base GlcNAcs. Once this reaction is complete, an EndoS mutant conjugated to the desired final glycan structure is used to transglycosylate the original molecule. Both enzymatic transformations are carried out under mild conditions and progress to completion within hours. Since the natural glycoform is cleaved, the process can be used on proteins produced by any alternative host. Separating production of glycans from protein production, however, does not scale well and could significantly increase overall manufacturing costs for conventional monoclonal antibodies. More efficient methods for the production of glycans as a raw material would be required for this approach to be widely adopted at large scale (Seeberger & Werz, 2007).

Alternatively, host glycoengineering uses enzyme knock-ins or knock-outs to enable the host organism to produce desired glycoforms. Like enzymatic glycosylation, this strategy typically leads to more homogeneous glycoforms than those produced naturally by CHO. *P. pastoris* was the first host to be successfully engineered to generate homogeneous glycoforms on antibodies that incorporate all five sugars (Gerngross, 2006; Jacobs, Geysens, Vervecken, Contreras, & Callewaert, 2009). The same approach has been applied successfully to other yeast and fungi (Figure 1b). Production of mammalian-like N-glycans has more recently been demonstrated in *T. reesei* (Natunen et al., 2015), with galactose and fucose but not sialic acid incorporated. *H. polymorpha* has been engineered to express human-like glycans with terminal galactose (Cheon, Kim, Oh, Kwon, & Kang, 2012; Wang, Song, Wang, & Qiu, 2013). The first steps of this process have also been engineered into *Aspergillus* and *K. lactis* (Kainz et al., 2008; Liu et al., 2009). Applying these glycoengineering strategies will likely be straightforward for *P. tricornutum*, *T. thermophila*, and *L. tarentolae*, which have natural glycan structures more similar to the human glycoforms. The intrinsic cellular organization of eukaryotic microorganisms dramatically simplifies glycoengineering compared to bacterial systems. While the steps of glycosylation that take place in the ER have been engineered into *E. coli*, the glycosylation efficiency is only 1% (Valderrama-Rincon et al., 2012).

Potentially simplifying the glycoengineering challenge, the complex and mixed glycoforms generated by CHO are not always necessary for clinical applications. Afucosylated variants of IgG1 showed improved binding to human FcγRIII compared to the fucosylated version, which translated into improved antibody-dependent cellular toxicity (ADCC) in vitro (Shields et al., 2002). Simple non-branched glycoforms generated in mammalian cells through the GlycoDelete method were demonstrated to have comparable efficacy in vitro and in mice (Meuris et al., 2014). Further, mouse IgG2c and human IgG1 and IgG3 subclasses of antibodies with only mono- or disaccharide glycosylation structures were shown to remain fully functional in vivo (Kao et al., 2015). Non-glycosylated variants of immunoglobulin G (with two to three mutations around the glycosylation site) displayed comparable effector function to the original glycosylated protein (Sazinsky et al., 2008). Further clinical studies will be required to validate safety and efficacy of different glycoforms in humans, but this area shows great promise for simplifying and lowering the costs of manufacturing processes both in CHO and alternative hosts.

N-linked glycosylation remains the post-translational modification of greatest interest because of its importance for antibodies, but other post-translational modifications are important for other protein therapeutics. Fortunately, these processes and the enzymes involved are also well-understood and can likely be addressed by analogous strain engineering. For example, yeast have been engineered to perform gamma-carboxylation, a PTM required for certain blood factors (Steenstrup, 2009). Human-like O-linked glycosylation, modifying serine or threonine residues, has also been engineered into *P. pastoris* (Hamilton et al., 2013). *T. reesei* has been engineered to reduce O-mannosyltransferase activity with no negative impact on growth (Natunen et al., 2015).

Although glycosylation factored into the initial selection of CHO as a host for therapeutic proteins, generation of human-like glycoforms is feasible in eukaryotic microorganisms and is likely to be accelerated by increased understanding of the therapeutic effectiveness of simpler glycoforms. Enabled and accelerated by the powerful strain engineering advances described in the previous section, glycoengineering should not be considered a significant barrier for eukaryotic microbial expression systems.

5 | FERMENTATION PERFORMANCE

Once a strain is engineered to produce the desired therapeutic protein, a fermentation process must be developed. The productivity of cell culture is determined by the cell growth and the specific productivity of each cell. While protein titer is often used to compare fed-batch processes of similar lengths, the adoption of perfusion for cultivation has highlighted the importance of volumetric productivity, measured in g/L/day. High productivity is a key requirement for both low production costs and very high volume production, addressing both unmet needs. The challenge of serving very large patient populations at reasonable cost may require an order of magnitude increase in productivity, from ~0.5 to ~5 g/L/day.

For very-high volume processes, one must consider the biological limitations on the productivity of CHO cultures. Increases in the productivities of CHO cultures to date have been achieved primarily through increases in cell density, while per cell productivity has been consistent in recent years at 15–30 pg/cell/day (pcd). Even at an ambitious 20 pcd and 50% downstream yield, reaching 5 g/L/day would require cell density of 500×10^6 cells/ml. CHO cells at this density would take up over 75% of the reactor volume, creating a

formidable bioprocessing challenge in both cell culture and product recovery. In contrast, eukaryotic microbial cells are much smaller so their volume fraction would be less than 10% at the same cell density.

As eukaryotic microorganisms grow faster than CHO cells, they already have an advantage in volumetric productivity based on their shorter outgrowth times. To grow from 10,000 to 10,000,000 cells/ml takes under 2 days in fungi, but 7 days in CHO (Fernández & Vega, 2013; Wurm, 2004). The faster outgrowth raises the theoretically achievable productivities and could dramatically reduce cycle times within facilities.

Several species of yeast and fungi have already been demonstrated to produce high levels of enzymes and therapeutically relevant proteins at bioreactor scale (Figure 2). Titers are lower than those of CHO, but volumetric productivities are comparable because the fermentations take less time. Industrial productivities of enzymes not fully described in the literature may be even higher, with titers of 100 g/L reported for some molecules (Cherry & Fidantsef, 2003). While these productivities will not translate directly to new molecules such as antibodies, they reflect the fermentation potential for these organisms. Additionally, strain engineering to improve productivity is expected to be significantly less expensive for alternative hosts because of their smaller genomes as described above. Process development strategies that have been developed with CHO cells, such as automated screening and statistical experimental designs, should also allow faster development of intense fermentation processes.

There are limited data for productivity from protozoa at bioreactor scale to date, but several companies have built their businesses around these organisms as expression hosts. Cilian AG markets their CIPEX system, a *T. thermophila* cell line, and has demonstrated expression of human enzymes, vaccines, and a monoclonal antibody. TetraGenetics, Inc., is developing antibody drugs to treat autoimmune conditions and pain using a *T. thermophila* host. Jena Biosciences sells kits for the LEXSY expression system, based on *L. tarentolae*. As more proteins are successfully demonstrated in these organisms, it is reasonable to expect that fermentation scale-up will follow soon thereafter.

In microalgae, large-scale production systems have been developed for biofuel applications rather than therapeutic protein production to date. Advances in open ponds and photobioreactors have demonstrated that high cell densities are feasible in controlled environments (Specht et al., 2010). For production of therapeutic proteins, heterotrophic growth on fed carbon sources instead of sunlight is likely a more promising approach, as discussed in a recent review (Perez-Garcia, Escalante, de-Bashan, & Bashan, 2011).

Adoption of eukaryotic microorganisms may also reduce fermentation costs. Fungi can be cultivated on lower quality carbon sources that could reduce costs of raw materials for fermentation and allow broader sourcing thereof, which in turn improves sustainability of supply. Industrial processes for enzymes regularly use agricultural waste as a feed source for fungi, and growth of *P. pastoris* has been demonstrated on industrial-grade glycerol (Çelik et al., 2008; Singhania et al., 2010). *K. lactis* can grow on lactose-containing waste such as whey from the dairy industry, expanding the range of substrates

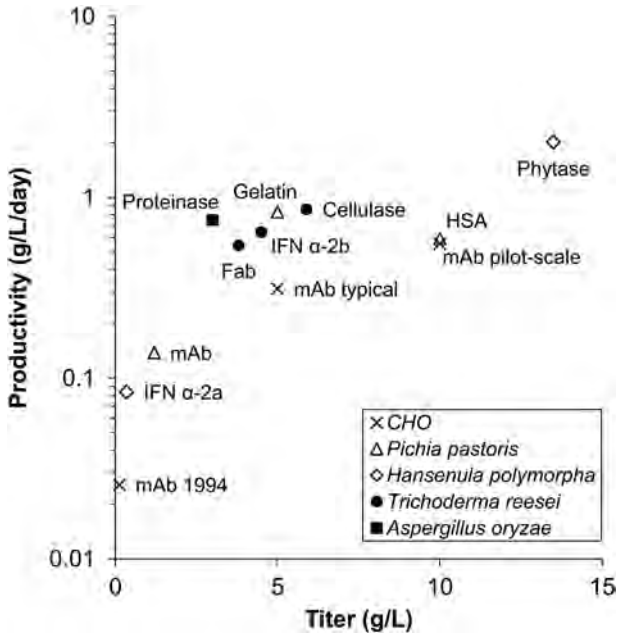

FIGURE 2 Comparison of fermentation performance for heterologous proteins across different hosts. Productivity was calculated from reported titer, outgrowth time, and production time. Yield was calculated from titer and carbon source consumption based on reported feed rates and durations. Values are based on data from CHO (Huang et al., 2010; Keen & Rapson, 1995), *P. pastoris* (Mallem et al., 2014; Potgieter et al., 2009; Werten, Van Den Bosch, Wind, Mooibroek, & De Wolf, 1999), *H. polymorpha* (Mayer et al., 1999; Müller et al., 2002), *T. reesei* (Landowski et al., 2016; Ma et al., 2013; Nyyssönen et al., 1993), and *A. oryzae* (Ward et al., 1995). With further development, alternative hosts could also see the order-of-magnitude improvement in fermentation performance that has been achieved in CHO. Calculations are based on detailed fermentation data published in peer-reviewed journals and do not reflect industry statements about highest achievable titers. Data only include cultivations in bioreactors using batch or fed-batch mode at over 1 L scale

further (Rodicio & Heinisch, 2009). Robustness to lower cost carbon sources could result in lower media costs that reduce the overall cost of production.

Eukaryotic microorganisms also tend to be more robust to environmental perturbations, which can lead to the design of lower cost processes. Fungal cells are less sensitive to shear than CHO, and thus can be mixed faster or with more force; this feature is a key factor driving their adaptability to 300,000 L bioreactors. Further work is required, however, to identify the "sweet spot" of mixing for filamentous fungi to maximize productivity (Grimm, Kelly, Krull, & Hempel, 2005). Eukaryotic microorganisms are also believed to be less sensitive to changes in temperature and pH than mammalian cells, but further work is required to directly compare these attributes.

Alternative hosts offer greater flexibility in mode and scale, so the greatest gains in cost or productivity may not result from using them directly as a replacement in the existing CHO platform. Opportunities may exist to circumvent traditional outgrowth paradigms, such as serial passaging of yeast, where one batch's cell mass is transferred to a

freshly loaded bioreactor. This technique has been used effectively in the brewing industry for 8–15 batches per production cycle (Powell & Diacetis, 2007). New harvest options also offer great potential to eliminate process steps and reduce costs. Several species studied here can be induced to flocculate: *P. pastoris*, *K. lactis*, *P. tricornutum*, and *C. reinhardtii* (Bellal, Boudrant, Elfoul, & Bonaly, 1995; Schlesinger et al., 2012; Sirin, Trobajo, Ibanez, & Salvado, 2012; Tanino, Fukuda, & Kondo, 2006). In the long term, one could imagine a single-vessel process where cells are grown and flocculated out and the protein is purified through crystallization. Further research is required for any of these approaches, but it is undoubtable that introducing alternative hosts opens up new process options that are simply not feasible with the current CHO-based processes.

6 | PURIFICATION PROCESS

Although the host organism is not directly involved in the downstream process, features of the host have implications for the complexity and nature of the purification process. Current purification processes may represent up to half of the total production cost and become a bottleneck as productivities increase (Kelley, 2009). A key requirement for low-cost purification is a clean feed; specifically, cell lysis should be avoided wherever possible. While lysis is required for *E. coli*-based processes, current CHO-based processes rely on secretion to separate the desired protein from the cell mass.

Secretion has been demonstrated in methylotrophic yeast, fungi, microalgae, and protozoa. Signal sequences are incorporated at the N-terminus of the protein of interest and direct the protein into the endoplasmic reticulum (ER) for folding and secretion. Both homologous and heterologous sequences have proven effective. The methylotrophic yeast secrete protein more effectively than *S. cerevisiae* because they have a stacked Golgi apparatus adjacent to the ER, better organized to facilitate secretion (Preuss, Mulholland, Franzusoff, Segev, & Botsteint, 1992). Biological studies and strain engineering, however, have shown that there is potential to improve other bottlenecks in secretion by *P. pastoris* (Idiris, Tohda, Kumagai, & Takegawa, 2010; Panagiotou et al., 2011). In contrast, filamentous fungi are very effective secretors. The relative fraction of their endogenous proteases secreted alongside the protein of interest,

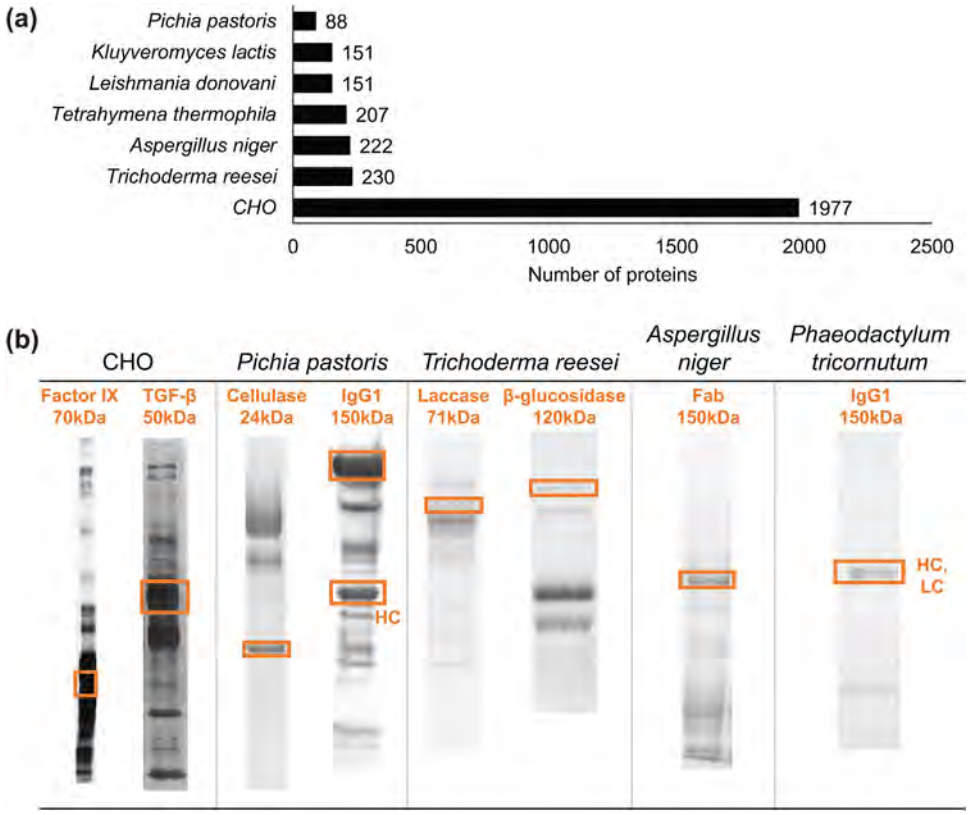

FIGURE 3 Factors relevant to purification of secreted proteins from culture supernatants. (a) Secretome sizes of host organisms. For species where data were not available, related species were included. Counts of distinct proteins were determined experimentally using mass spectroscopy (Adav, Chao, & Sze, 2012; Baycin-Hizal et al., 2012; Madinger et al., 2009; Madinger, Collins, Fields, Taron, & Benner, 2010; Mattanovich et al., 2009; Tsang, Butler, Powlowski, Panisko, & Baker, 2009). (b) Comparison of SDS–PAGE gels of supernatants from heterologous protein production stained with Coomassie blue, recolored in black and white. Only gels run on unpurified material were included. The band for the target protein identified by the authors is boxed in orange. For IgG1, bands of cleaved heavy chain (HC) and light chain (LC) were also boxed. Images adapted for CHO (Gentry et al., 1987; Kaufman, Wasley, Furie, Furie, & Shoemaker, 1986), *P. pastoris* (Potgieter et al., 2009; Várnai et al., 2014), *T. reesei* (Kiiskinen et al., 2004; Ma, Zhang, Zou, Wang, & Zhou, 2011), *A. niger* (Ward et al., 2004), and *P. tricornutum* (Hempel & Maier, 2012)

however, can be high, and can cause product degradation. Knocking out these proteases has been demonstrated to increase titers without eliminating cell growth (Landowski et al., 2015). Secretion signals have been used in *L. tarentolae* and secretable granules are produced in *T. thermophila* (Basile & Peticca, 2009; Jayaram et al., 2010). Antibody secretion has been demonstrated at low titer in diatom *P. tricornutum* (Hempel & Maier, 2012; Vanier et al., 2015). In *C. reinhardtii*, secretion of protein has only been demonstrated efficiently in cell wall-deficient strains (Eichler-Stahlberg, Weisheit, Ruecker, & Heitzer, 2009).

Evidence suggests that fungi and protozoa secrete a much lower load of host cell proteins than CHO cells. The number of different proteins secreted by CHO is an order of magnitude larger than that of eukaryotic microorganisms for which data were available (Figure 3a). Additionally, the purity of the protein of interest is much higher for yeast and fungi than in raw CHO supernatants (Figure 3b). These less complex feedstocks may enable wider use of new purification technologies such as multimodal resins or membrane chromatography. Since comparable data are not available for many eukaryotic microorganisms, data directly comparing all of these organisms would be very informative for future host selection.

The safety profile for alternative hosts may offer certain advantages when compared to CHO, although more study is required in this area. In CHO-based production processes, viral inactivation is an important and expensive step because some viruses that present a risk to humans can also propagate rapidly in CHO (Berting, Farcet, & Kreil, 2010). In contrast, eukaryotic microorganisms are not susceptible to mammalian viruses, so adventitious agents that pose a risk to humans would not be able to propagate and the viral clearance requirements could be reduced. In the European Medicines Agency (EMA) assessment of Jetrea (Ocriplasmin), produced in *P. pastoris*, no raw materials of animal or human origin are used in the manufacturing process so there was agreement of reduced risk of contamination with viral adventitious agents (EMA/CHMP/74766/2013). A similar approach could be used for therapeutics made in other eukaryotic microorganisms.

Several of these organisms also have established safety profiles relevant for biologic drug production. FDA-approved drugs manufactured in *S. cerevisiae* include insulin and insulin analogs, hirudin, growth hormone, and vaccines (Walsh, 2014). Over 10 drugs produced in *Pichia* have been approved worldwide for parenteral use, including Dyax's Kalbitor, ThromboGenics' Jetrea, Shanvac hepatitis vaccine, albumin, insulin, interferon-alpha, and two antibody fragments (http://www.genengnews.com/gen-articles/i-pichia-pastoris-i-revisited/5230/). While approved drugs have not been produced in the other host organisms, some safety information is already available. Enzymes made using *T. reesei*, *Aspergillus niger*, *A. oryzae*, *H. polymorpha*, and *K. lactis* have been used in food production and are already on the FDA's Generally Recognized as Safe (GRAS) list. Seaweeds and alginates are also GRAS, suggesting microalgae could also have suitable safety profiles. Although *Leishmania* is a parasite, the *L. tarentolae* species is not pathogenic in humans (Basile & Peticca, 2009). Further research is required for many microorganisms to establish toxicology profiles and evaluate potential risk, but the use of modern analytical technologies for assessing product quality will facilitate risk assessment.

TABLE 2 Summary of characteristics relevant to use as biomanufacturing host for each class of eukaryotic microorganism.

Host	Strengths	Challenges
Yeast		
Saccharomyces cerevisiae	Proven expression of mAbs	Specific productivity
Pichia pastoris	Well-established genomes Rapid growth rate	
Hansenula polymorpha	Robust phenotype	
Kluyveromyces lactis	Vast public knowledge	
Filamentous fungi		
Trichoderma reesei	Proven expression of mAbs	Proteases
	Well-established genomes	
Aspergillus oryzae	High per-cell productivity	
	Vast public knowledge	
Microalgae		
Chlamydomonas reinhardtii	High per-cell productivity	Cell culture
Phaedactylum tricornutum	Proven expression of mAbs	
	High per-cell productivity	
Protozoa		
Tetrahymena thermophila	Proven expression of mAbs	Genomic complexity
Leishmania tarentolae	Complex glycans	Robustness

To harness the full benefits of using alternative host organisms in the upstream process, the bioprocess community must develop downstream processes that are tailored to the properties of these microbial feedstocks rather than adopting platforms tailored for CHO-based processes. The lower loads of host cell proteins and lack of mammalian viruses present an opportunity to simplify unit operations and consider new modes of purification that could scale more easily and be operated at lower cost. For example, the large number of chromatography steps required for non-mAb purification (Brower et al., 2014) could potentially be reduced if the initial feed has higher purity. If high molecular weight impurities are at low enough concentrations, preparative crystallization of proteins may offer significant economic advantages compared to chromatography (Hekmat, 2015). Most proteins produced in alternative hosts to date have been purified using conventional chromatographic schemes, so there is limited understanding of the potential improvements to yield and economics. Further study is encouraged in these areas going forward, in parallel with the development of the host organisms.

7 | CONCLUSIONS AND OUTLOOK

Alternative host organisms, and the new manufacturing processes they enable, provide hope for two unmet needs in production of therapeutic proteins: extremely low-cost production and extremely high-volume production. The host is the fundamental unit that drives the surrounding manufacturing process for recombinant proteins. A fast-growing host with a simple genome could accelerate strain engineering and allow for significant advances in developing cells as "protein factories." Strain development will be required to increase productivity for all alternative hosts, but this process will be dramatically accelerated by faster growth and better biological understanding than when CHO was developed as a host. Eukaryotic microorganisms can be grown in larger tanks, enabling even greater economies of scale, and have the potential to realize greater productivities through faster doubling times and reduced cycle times within facilities. The lower loads of host cell proteins and limited viral burden may allow for simpler and lower cost purification processes.

Although the knowledge base about alternative host organisms has grown significantly, additional research is still required to further explore their capabilities and improve their performance. Each of the eukaryotic microorganisms explored here has strengths that make it well-suited to therapeutic protein manufacturing, but also challenges (Table 2). Antibody expression and intensive glycoengineering have already been demonstrated for yeast and filamentous fungi, which have been more heavily studied. Questions still remain, however, about the best strategies to increase specific productivity in yeast. For filamentous fungi, protease activity still poses a risk to consistent production of high-quality proteins and simplified purification with few product-related variants. Microalgae display promising per-cell productivity, but require either a transition to photobioreactors or development of strategies for heterotrophic growth in traditional cell culture formats. Protozoa can produce complex proteins and favorable glycans, but *T. thermophila* has a very complex genome and *L. tarentolae* is comparatively unproven. Other species of eukaryotic microorganisms from these classes may provide additional opportunities for therapeutic protein production if they are developed further as expression hosts.

A comprehensive comparative study would help address some of the gaps highlighted throughout this review and enable informed selection of host organisms for different therapeutic protein targets. While this review has focused on engineering required for high productivities, product quality beyond glycosylation is also critical to host success. For example, C-terminal sequence variability is commonly observed in CHO-derived antibodies (Dick, Qiu, Mahon, Adamo, & Cheng, 2008). Protein sequence variants and other quality attributes will need to be studied in more depth and evaluated for clinical impact. Further study is also required to address concerns of potential toxicity or immunogenicity for some of these organisms, including studies of host cell proteins.

As a regulated industry, biopharmaceutical manufacturing faces barriers to potentially risky innovation that can be lowered through creative funding models. A cooperative effort could stimulate the research required to bring one or more alternative hosts to industrially relevant performance standards, just as collaboration and open access facilitated the early development of CHO as a host organism. Successful development of alternative hosts could enable the industry to expand access to less privileged patient populations and large patient populations whose needs may not be met with the current paradigm for bioprocess.

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