Sunflower

Application Note

Microbial Production

Keywords

Bioreactor, perfusion fermentation, protein manufacturing, yeast, defined media

Chemically Defined Media for Yeast

STx-001 supports ultra high biomass and production of many proteins from yeast

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Abstract

Sunflower has recently developed STx-001, a commercially available rich defined media that enhances fermentation of yeast by enabling high biomass and production of a variety of protein classes. STx-001 is fully chemically defined, thereby filling a key gap in the market for fermentation media ready to use in regulated environments. Here, we demonstrate the versatility of STx-001 in both batch and perfusion fermentation modes to achieve improved space time yields of a variety of recombinant proteins.

Introduction

Yeast, such as *Pichia pastoris*, are increasingly being used for recombinant protein production in both industrial fermentation and biopharmaceuticals. This is due to several key benefits of yeast compared to bacteria or mammalian cell hosts, including the ability to secrete complex proteins at >70% initial purity; fast growth rates; small, tractable genomes; and lack of contamination with endotoxin or adventitious agents. Commercially available media for yeast fermentation, however, is limited. Buffered complex media (BMxY) is often used in routine batch and fed-batch cultivations, but these media contain complex components, such as hydrolysates, that can cause significant lot-to-lot variability and are highly discouraged in regulated environments (1). A few minimal defined media formulations that do not contain complex components are also commonly used, such as basal salts medium (BSM). These minimal defined media often show lower growth rates and reduced expression compared to complex media. Recently, Matthews et al. developed a rich defined media specifically for yeast that resulted in growth and protein production at similar or higher levels than complex media, but the concentration of such rich defined media to support high biomass is limited due to precipitation (2).

Sunflower has developed a proprietary rich defined media (STx-001) for fermentation of yeast, such as *P. pastoris*, that can: 1) sustain high biomass 2) support production of a variety of protein classes and 3) be concentrated for use with different modes of fermentation, like batch, fed-batch or perfusion fermentation. Sunflower's media formulation contains a proprietary blend of amino acids, vitamins, salts, and other essential nutrients optimized for yeast fermentation. Key advantages of STx-001 are summarized in Table 1.

TABLE 1: Key characteristics of STx-001 compared to existing media formulations.

	STx-001	Complex Media	Basal Salts Media	Rich Defined Media[2]
Chemically Defined	✓	✗	✓	✓
Supports concentrated formulation	✓	✗	✗	✗
Enables high growth rates	✓	✓	✗	✓
Supports ultra high cell density	✓	✗	✗	✗
Supports production of a variety of protein classes	✓	✓	✓	✓
Filter sterilizable	✓	✓	✓	✓

Materials and Methods

Bench-scale comparison of STx-001 & other commercially available media

P. pastoris strains expressing several different recombinant proteins were cultivated in 10 mL deep-well plates (3 mL working volume) in either STx-001, commercially available complex, or basal salts media. Biomass accumulation was conducted in glycerol-containing media for 24 - 48 hours at room temperature with agitation at 600 rpm. Then, the glycerol-containing media was exchanged for methanol-containing media for each formulation (complex or defined) for an additional 24 - 48 hours to promote recombinant protein expression. Cultivations conducted in 1L flasks (200 mL working volume) were incubated at 25° C with shaking at 250 rpm. Biomass was measured by absorbance at OD_{600nm} and protein expression was confirmed using SDS-PAGE and/or product-specific western blot.

Performance of STx-001 in perfusion fermentation

A single-domain antibody (V_HH) was produced via perfusion fermentation in the Daisy Petal™ bioreactor using a strain of *P. pastoris* engineered by Sunflower as described previously (3). Glycerol-containing STx-001 at 1X concentration was continuously fed for the first 24 hours, glycerol-containing STx-001 at 2X concentration (including 2X glycerol) was fed during hours 24-48, and then methanol-containing STx-001 at 2X concentration was fed for hours 48-290 to induce protein expression (approximately 10 days of methanol induction). Wet cell weight (g/L) was determined using cell-containing samples from inside the bioreactor. Protein titer and yield was determined by SDS-PAGE using samples of the cell-free harvest fluid.

Results

STx-001 supports high cell density at bench-scale

To test the capacity of STx-001 to support high cell density in plate-scale cultivations, three different engineered *P. pastoris* strains were grown in either STx-001 or commercially available complex media, both containing glycerol, for 48 hours (Figure 1). After 24 hours, all strains reached similar OD_{600nm} in both media. After 48 hours, strains in STx-001 reached roughly twice the biomass concentration compared to strains grown In the commercially available complex media, demonstrating the improved

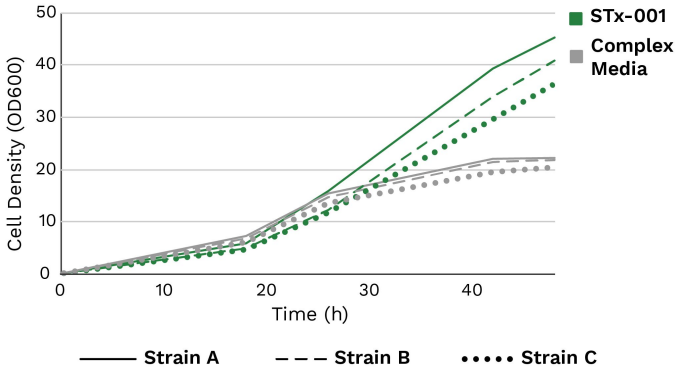

FIGURE 1: Cell density of three different engineered P. pastoris strains grown in different media over time.

capacity of STx-001 to support high biomass at plate-scale.

STx-001 supports productivity of diverse protein classes

Methanol-containing STx-001 was also tested to demonstrate the production of a variety of recombinant proteins. Production of a single-domain antibody was compared in STx-001, complex media, and basal salts media in a flask-scale cultivation (Figure 2A, B). Recombinant protein-expressing *P. pastoris* was grown in glycerol-containing media for 24 hours, then exchanged into methanol-containing media for an additional 48 hours to promote protein production. Growth and productivity were similar in STx-001 as compared to the complex media, and growth and productivity were the lowest in basal salts media.

Protein production was also evaluated using a *P. pastoris* strain engineered to express a single-domain antibody under a glycerol derepression promoter system (i.e. methanol-free protein production). In this case, cells were grown in STx-001, complex media, or basal salts media for 24 hours with 4% glycerol, then exchanged into the same media background containing 1% glycerol for 48 hours to promote protein production. An additional spike of 1% glycerol was added after 24 hours of production. For the methanol-free expression system, cultures in STx-001 produced significantly more protein compared to complex or basal salts media (Figure 2C).

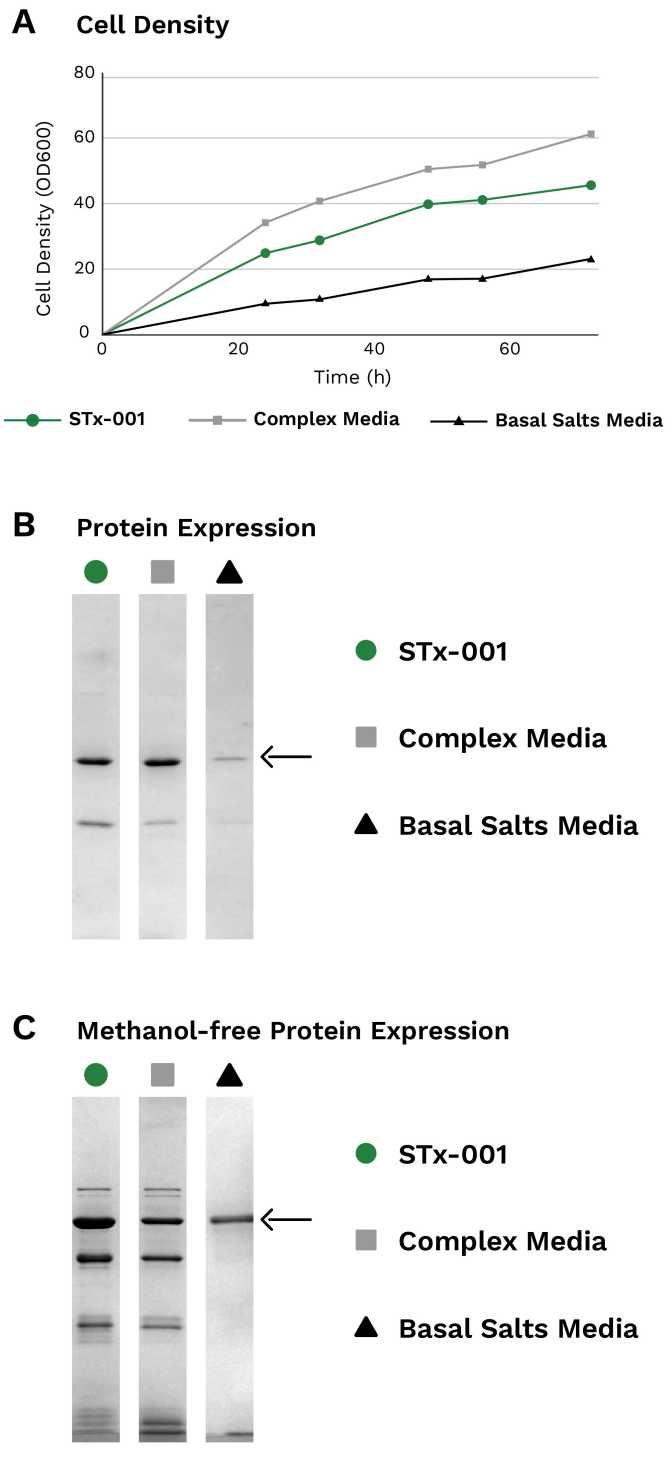

FIGURE 2: Cultivation of P. pastoris expressing a single-domain antibody (arrow) using methanol (A, B) or methanol-free (C) induction in STx-001 (green), complex media (gray), or basal salts media (black). A. Cell density over time. B and C. SDS-PAGE of unpurified cultivation samples.

Recombinant protein production was further demonstrated in STx-001 for yeast strains expressing monoclonal antibodies, single-domain antibodies, Fc-fusions, and virus-like particles (Figure 3).

STx-001 supports perfusion fermentation in bioreactors

STx-001 was then tested for its ability to support high biomass and protein production in perfusion fermentation using Sunflower's Daisy Petal™ Perfusion Bioreactor System (Figure 4). A *P. pastoris* strain expressing a recombinant single-domain antibody was used. Concentrated STx-001 (up to 2X) was used to support the significantly higher biomasses achievable in bioreactors (five to ten times higher) as compared to plate- or flask-scale cultivation. To build biomass, glycerol-containing STx-001 (1X) was fed for the first 24 hours, and then glycerol-containing STx-001 (2X) was fed for the next 24 hours. After 48 hours, methanol containing STx-001 (2X) was fed for the remaining 242 hours. A wet cell weight of >400 g/L was reached at the end of outgrowth and the wet cell weight was maintained between 400 - 600 g/L throughout the production phase. Protein titer in the continuous bioreactor ranged between 150 - 200 mg/L throughout production, resulting in the collection of over three grams of protein.

Preliminary data from fed-batch fermentations also demonstrates that concentrated STx-001 (up to 3X concentration) can yield more than 5-fold higher titers compared to complex media (unpublished data).

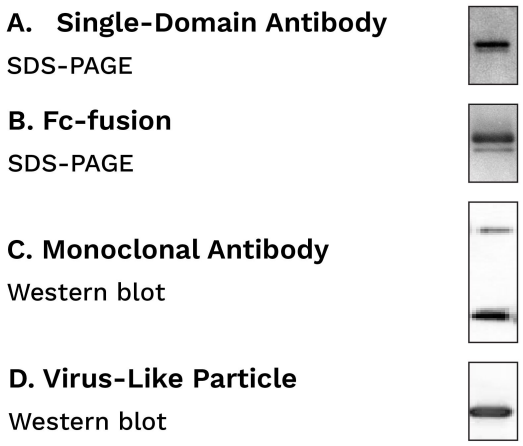

FIGURE 3: SDS-PAGE (A, B) or product-specific western blot (C, D) for recombinant proteins produced from cultivation of P. pastoris in STx-001.

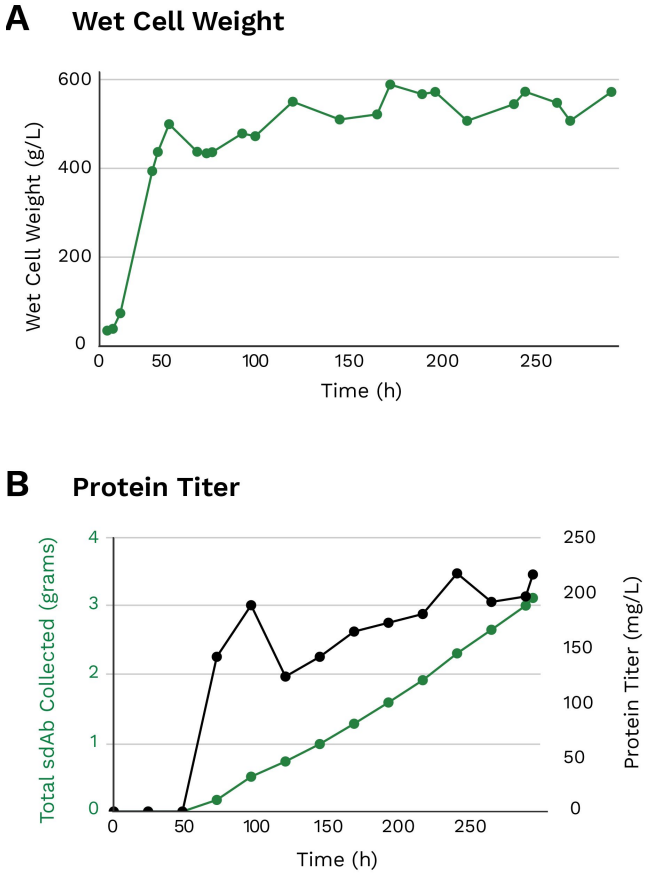

FIGURE 4: Perfusion fermentation of P. pastoris expressing a single-domain antibody in STx-001. A. Wet cell weight. B. Protein titer and cumulative protein collected as measured by densitometry on SDS-PAGE.

Conclusion

STx-001, Sunflower's proprietary defined media formulation for yeast, was demonstrated to support high cell density cultivation and recombinant protein production across a variety of protein types, scales and fermentation modes. The ability to produce different concentrations of the base media formulation without significant precipitation for different purposes was also demonstrated, which is a significant advantage for users interested in generating a high biomass in diverse fermentation applications. Using a highly soluble defined media formulation enables scaling of the nutrient concentration to better address the biomass levels achievable at different scales and with different fermentation methods.

Benefits of STx-001

- ✓ 100% chemically defined
- ✓ No hydrolysates or animal-origin productions
- ✓ High stability of concentrated formulations
- ✓ Supports high cell density cultures across multiple scales and fermentation modes
- ✓ Supports expression of proteins across a wide variety of protein classes
- ✓ Filter sterilizable

References

1. van der Valk, J. et al. Optimization of chemically defined cell culture media – Replacing fetal bovine serum in mammalian in vitro methods. *Toxicol. In Vitro* **24**(4), 1053-1063 (2010).
2. Matthews, C. B. et al. Development of a general defined medium for Pichia pastoris. *Biotechnol. Bioeng.* **115**(1), 103-113 (2018).
3. Crowell, L. et al., "Perfusion fermentation in the Daisy Petal™ bioreactor" (Sunflower Therapeutics 2024 Application Note).

Related Resources

Application Note: Perfusion fermentation in the Daisy Petal™ bioreactor

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