

Cultivation of aggressive microbial cultures of *Pichia* and *Bacillus* using the HyPerforma 30 L and 300 L Single-Use Fermentors

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Introduction

Advances in single-use bioprocess technology have led to the rapid adoption of single-use bioreactors (S.U.B.s) across a broad range of applications within the biotechnology industry. However, the aggressive performance demands common in industrial microbiology have limited the conversion of traditional fermentation processes to single-use systems. To address the unique needs of microbial applications, we created the Thermo Scientific™ HyPerforma™ Single-Use Fermentor (S.U.F.) as the first truly scalable single-use, stirred-tank reactor system utilizing proven scale-up principles (Figure 1). Offered for both 30 L and 300 L working volumes, both systems are designed to meet the most rigorous process demands of high-performance mixing and mass transfer. Existing fermentation production facilities can retrofit the S.U.F. in place of traditional stainless steel cleaning-in-place/sterilization-in-place (CIP/SIP) fermentor vessels without modifying their procedures. As expression systems are moved to production volumes, they can easily be scaled from a benchtop fermentor (or flask) to the S.U.F. A significant benefit of the S.U.F. when compared to existing single-use offerings is that the product is specifically built for the microbial process and offers very efficient and simple technical transfer—the S.U.F. is not simply modified from existing cell culture reactors.

Many aggressive microbial cultures require systems that can produce an oxygen transfer coefficient ($k_L a$) greater than 360 h^{-1} (as measured without supplementing oxygen). These high $k_L a$ values often require gas flows of two vessel volumes per minute (vvm). Single-use systems have constrained gas flow rates due to pressure safety limits of the single-use flexible container. The required low operating backpressure decreases the gas flow capacity of exhaust filters. To



Figure 1. HyPerforma 30 L and 300 L Single-Use Fermentors with condensers.

overcome this, we have developed a single-use exhaust filter and condenser system rated to allow 2 vvm of gas flow over two weeks of operation. Higher flow rates may be achieved by simply adding more filter modules or shortening the duration of the culture. In this report, we demonstrate various cultivations at high gas flow, seeding at 20% working volume, and a comparison of the S.U.F. to traditional stainless steel fermentors. The HyPerforma S.U.F. is designed to be a fermentor and thus meets the performance requirements of dense, rapidly growing microbial cultures while offering the benefits of quick process setup, reduced contamination risk, and high production quality of the original Thermo Scientific™ HyPerforma™ Single-Use Bioreactor (S.U.B.).

Materials and methods

Design and process parameters

The standard S.U.F. configuration provides three Rushton impellers and quadrantal baffles in the vessel, as well as the standard aspect, spacing, and power-to-volume ratios common to most industry-scale and research-scale fermentors. Appropriate temperature control units (TCUs) were connected to each vessel and exhaust condenser. Vessel load cells were utilized to monitor the liquid mass in each vessel. A single-use capacitance probe (integral to the bioprocess container, or BPC) was used to control excess foam generation via the addition of an antifoaming agent (Antifoam C8840, New London Chemicals). Single-use C-Flex™ tubing allowed for feed connections using a tube welder (aseptic technique using quick connects is an option). Integral single-use pressure sensors (Pendotech) were used to monitor the process gas overlay pressure; a pressure interlock at 0.5 psi was utilized to ensure that the BPC was not overpressurized.

Cultivation procedure

Pichia pastoris glycerol- and methanol-fed batch cultures

Fed-batch cultures of *P. pastoris* strain GS115 expressing cutinase, a 22 kDa protein, were established in HyPerforma 30 L and 300 L Single-Use Fermentors. The initial seed culture was started 40 hours prior to S.U.F. inoculation from a single colony transferred from a YPD agar plate to YPD medium (containing Zeocin™ antibiotic). An initial seed of 8 mL was transferred to each liter of BMGY medium (one-tenth initial of S.U.F. volume) 20 hours prior to S.U.F. inoculation. Seed was cultured at 30°C and 250 rpm for 16 hours. OD₆₀₀ was recorded, and seed was transferred through aseptic connections to basal salt medium (BSM) containing 20 g/L glycerol in the S.U.F. Once glycerol was consumed, as indicated by a peak in the percentage of dissolved oxygen (DO), a 50% glycerol feed was used for 4 hours. When a second peak in DO was noticed after ending the glycerol feed, a 99% methanol induction feed was started. The feed was stepped up as needed for optimal protein expression for an additional 24 hours. See the document “*Pichia* Fermentation Process Guidelines” for additional details. [1]

The S.U.F. was operated with the following parameters. During cultivation, pH was controlled at 5.0 ±0.1 with 28% NH₄OH and 43% H₂SO₄. The DO level was set to 30% and controlled with the cascade setting, with stirrer ramped up first, then air, followed by oxygen. Oxygen was supplied from a liquid oxygen dewar through a 2,400 standard cubic feet per hour (scfh) vaporizer. If the DO fluctuated to below 20%, the cascade minimum set points were increased manually to avoid dropping the DO below 10%. The temperature was controlled at 30°C until just before methanol induction, when the temperature was set to 28°C. Samples were taken periodically for dry cell weight (DCW) and protein analysis. Protein yields were estimated using a densitometric method in which samples were run against BSA standards and the gel image was analyzed using TotalLab™ software. Media were hydrated in 25 L or 250 L batches using the Thermo Scientific™ HyPerforma™ Single-Use Mixer DS 300. Hydrated media were filtered through a 0.45 µm filter followed by a gamma-sterilized 2" 0.2 µm PVDF filter for the 30 L S.U.F. or a 5" 0.2 µm PVDF filter for the 300 L S.U.F.

P. pastoris exponential glucose-fed batch cultures

For initial *P. pastoris* cultures, modified YPD medium was used with a feed solution of 75% glucose and 4.35 mL/L PTM1 trace solution for the exponential feed. Modified YPD medium consisted of 10 g/L yeast extract, 20 g/L soy protein hydrolysate, 20 g/L dextrose, and 4 mL/L PTM1 trace solutions [1]. Exponential glucose-fed batch cultures of *P. pastoris* strain GS115 were grown in HyPerforma 30 L and 300 L Single-Use Fermentors. Seed culture was prepared 36 hours prior to fermentor inoculation by adding 1 mL of GS115 freezer stock to 2 L of filter-sterilized YPD. Seed was cultured at 30°C and 250 rpm for 16 hours. OD₆₀₀ was recorded, and seed was transferred through aseptic connections to 25 L of YPD in the HyPerforma 30 L S.U.F. For 300 L S.U.F. testing, the 30 L S.U.F. was used to prepare a 10-hour seed culture and then transferred to 300 L.

The S.U.F. was operated with the following parameters. During cultivation, pH was controlled at 5.6 ±0.1 with 28% NH₄OH and 5 M H₂SO₄. The DO level was set to 30% and controlled via the cascade setting, with stirrer ramped up first, then air, followed by oxygen. The temperature was controlled at 30°C. Samples were taken periodically for DCW and glucose analysis. Glucose was maintained by increasing the feed pump set point manually to maintain 15 ± 5 g/L glucose.

Inoculation at 20% working volume

Since the HyPerforma S.U.F. is designed to run at 20% volume (5:1), a 3 L seed could be used to inoculate 60 L in the 300 L S.U.F. This eliminated the need for a benchtop fermentor for the seed step, since 2 seed flasks could be used for the initial 60 L volume. Once the desired seed density was reached, additional media could be filtered in, to bring the total volume to 300 L.

Bacillus subtilis culture

To demonstrate 5:1 culture volumes, a high-yield *B. subtilis* culture was generated. Seed was initiated in 20 mL SRS medium using a single colony from an SRS agar plate. A second seed with M5 semi-synthetic rich medium was inoculated at 2.5% with overnight culture from the SRS medium seed. M5 medium (60 L) was sterile-filtered into the 300 L S.U.F., the medium was conditioned, and then 4 mL/L of 1 M MgSO₄, 3 mL/L of MKT trace elements solution, and 20 µg/mL of kanamycin were added prior to inoculation. The culture was inoculated at 5% of culture volume (3 L) with the second overnight seed. All *B. subtilis* cultures contained 20 µg/mL kanamycin. Standard procedures developed for a 10 L CIP/SIP vessel were followed for optimal protein expression. Air was manually ramped up to 300 standard liter per minute (slpm) as needed to support respiration demand.

P. pastoris glycerol- and methanol-fed batch culture

A *P. pastoris* culture was performed at 64 L pre-inoculation volume (20.3% of working volume). The above procedures for glycerol- and then methanol-fed *Pichia* cultures were followed for scaling up of cutinase production. The final culture volume was about 100 L (1/3 maximum working volume), with the same procedure for seed, feed strategy, and base solution feed used for pH control.

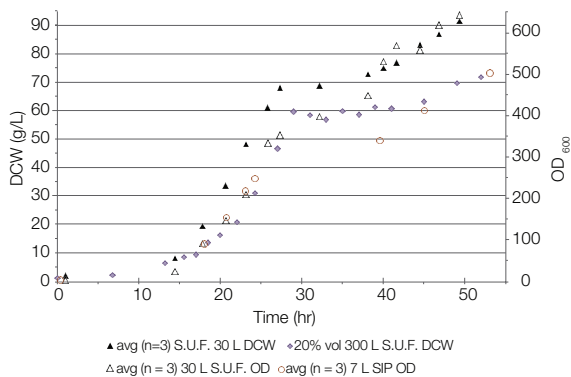


Figure 2. Plot of dry cell weight (DCW) and culture density for exponential-fed batch *P. pastoris* batch cultures from the 7 L SIP, and S.U.F. fermentors. The switch from glycerol feed to methanol feed is apparent at around 28 hours with the notable growth rate change. The final OD₆₀₀ of these cultures reached about 600.

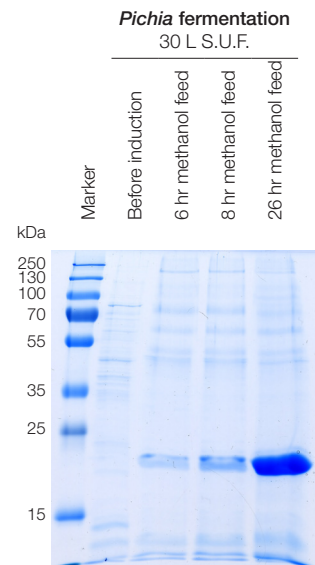


Figure 3. SDS-PAGE of supernatant samples from one 30 L S.U.F. *P. pastoris* culture. Protein gel images of the other *P. pastoris* cultures were similar. Cutinase protein yields from the three 30 L S.U.F. cultures were on average 1.6 ± 0.5 g/L.

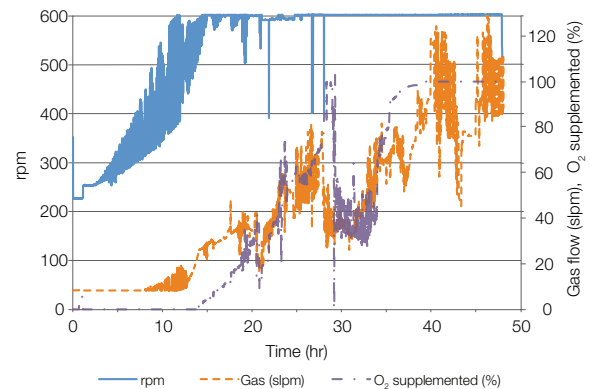


Figure 4. Graph of average (n = 3) agitation, total gas flow, and oxygen supplementation for three exponential-fed *P. pastoris* batch cultures in the 30 L S.U.F. Peak gas flow reached 5 vvm. The reduction in gas flow at around 28 hours is due to induction with the change from glycerol to methanol as the feed substrate. Flow rates were comparable to those performed in Sartorius 10 L stainless steel fermentor.

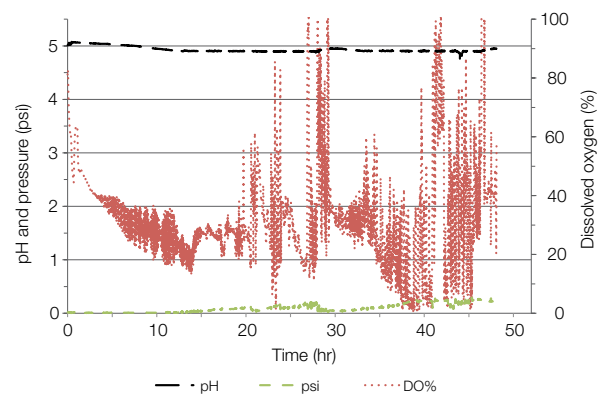


Figure 5. Graph of average (n = 3) pH, pressure, and DO of three 30 L *P. pastoris* cultures expressing cutinase. DO was not limiting, which was apparent due to continued exponential growth.

Results

P. pastoris glycerol- and methanol-fed batch cultures

An OD₆₀₀ of 659 was achieved with the first *P. pastoris* culture tested, with a final wet cell weight (WCW) of 303 g/L, a DCW of 95.6 g/L, and a cutinase yield of 2.1 g/L. Two more 30 L cultures and one 100 L culture were performed, reaching about the same density, dry cell weight, and cutinase yield. Gas flows up to 5 vvm were used with this rapidly growing, high-density culture.

The *P. pastoris* cultures started with BSM containing 2% glycerol and were fed glycerol for 4 hours once the initial glycerol was consumed. This was followed by 24 hours of methanol feed. The 30 L cultures achieved, on average (n = 3), a final OD₆₀₀ of 640, a WCW of 297 g/L, a DCW of 92 g/L, and a cutinase yield of 1.6 ± 0.5 g/L (Figure 2). Expression of target protein was apparent 6 hours after methanol induction, with yield increasing steadily thereafter (Figure 3). Gas flow reached up to five times the liquid volume during expression culture (Figure 4). Data for pH, pressure, and DO for the 30 L S.U.F. cultures are shown in Figure 5. Yields were similar to those for typical 7 L *P. pastoris* production runs in a Sartorius™ 10 L stainless steel SIP fermentor at our pilot-scale protein production lab (Figure 6).

The 20% working volume *Pichia* culture was performed at a 64 L initial volume in the 300 L S.U.F., ending with a 100 L culture volume. Air flow was switched off by incorrect DO cascade settings during the first hours of culture, unintentionally creating anaerobic conditions (DO levels decreased to 0% for several hours). Once the DO cascade settings were corrected, the culture recovered but with a longer initial lag phase and slightly lower cell densities. Data for the 20% working volume culture were shifted 3.5 hours to account for the increased initial lag phase attributed to no gas flow being supplied to the culture, which aligned the plots of exponential growth for comparison. The lower DCW trend may have been due to shifting to methanol following the normal feed procedure (Figure 2). However, 1.1 g/L of cutinase protein was produced, which was the same yield as that of one of the three 30 L S.U.F. cultures. For *P. pastoris* cultures, a WCW of 300 g/L and a DCW of 90 g/L were routinely achieved, with expression of methanol-induced protein typical of that of CIP/SIP fermentors.

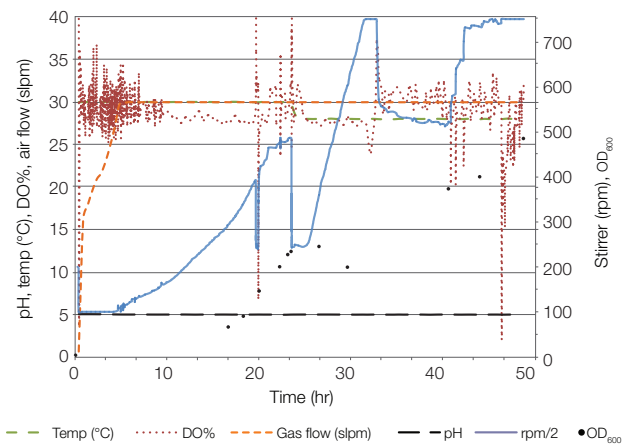


Figure 6. Graph of a routine 7 L glycerol- and then methanol-fed *P. pastoris* culture in a Sartorius 10 L stainless steel SIP fermentor. Air at 30 slpm was used starting at about 4:30 hours. Starting at around 34 hours, O₂ was supplemented to maintain the DO at a set point and reached 100% by 47 hours.

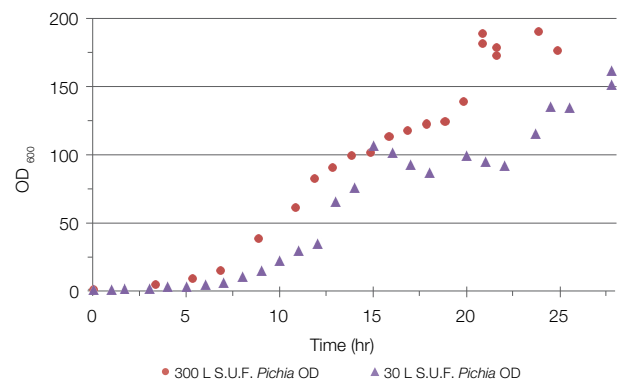


Figure 7. OD₆₀₀ plot for exponential glucose-fed *P. pastoris* batch cultures. The cultures were grown in YPD medium with glucose maintained at 2% with a 75% glucose feed.

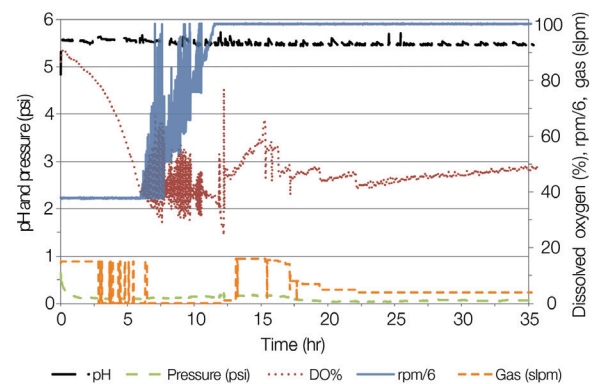


Figure 8. Glucose-fed 30 L *P. pastoris* batch culture. Oxygen was supplemented up to 1 slpm.

P. pastoris exponential glucose-fed batch cultures

For the 30 L S.U.F., the OD_{600} of the *P. pastoris* culture reached 161 ± 10 with a DCW of 21.26 g/L by 27:45 hours post-inoculation (Figure 7). The OD_{600} of the 300 L S.U.F. culture reached 190 ± 10 with a DCW of 20.83 g/L by 23:50 hours. DO was maintained above 30% with agitation and gas flow. The strains did not have expression sequences and thus were not induced. Figures 8 and 9 illustrate the similar respiration patterns achieved from 30 L to 300 L, which demonstrates the scalability of the process in the S.U.F.

20% working volume cultures

The 60 L working volume culture of *B. subtilis* had gas flow rates reaching up to 3 times the volume of the culture and speeds up to 350 rpm (Figure 10). The expressed 50 kDa protein yield was 0.4 g/L at 14 hours of culture. The culture had densities and protein production yields similar to routine *B. subtilis* high-yield cultures in a stainless steel Sartorius fermentor with a 7 L working volume (Figures 11 and 12).

20% of 300 L S.U.F. *P. pastoris* culture

As noted in the cell yield graph (Figure 2), a *P. pastoris* culture was performed at 64 L initial volume (20.3% of working volume). With added seed culture and feed solutions, the final culture volume was 100 L, a third of the maximum working volume for the 300 L S.U.F. The 300 L S.U.F. performed proficiently at 20% volume (5:1) and, as volume increased, to 33.3% with feed. Conditions and yield of the 20% cultures were similar to those of full vessels in both 10 L stainless steel and 30 L S.U.F. cultures.

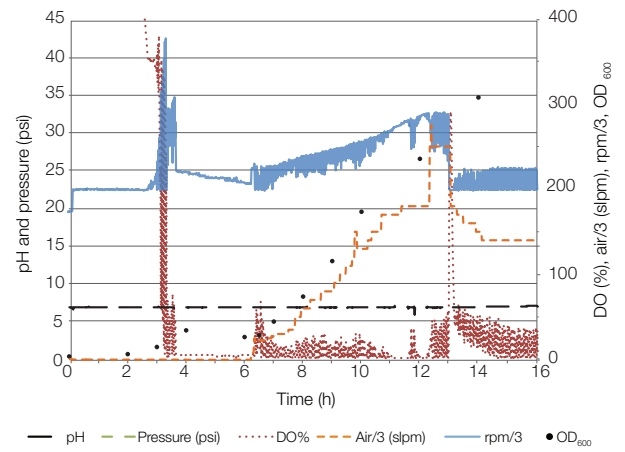


Figure 10. A *B. subtilis* culture growth in the 300 L S.U.F. at 20% working volume (60 L). Yields were comparable to those of typical cultures in a 10 L stainless steel SIP fermentor.

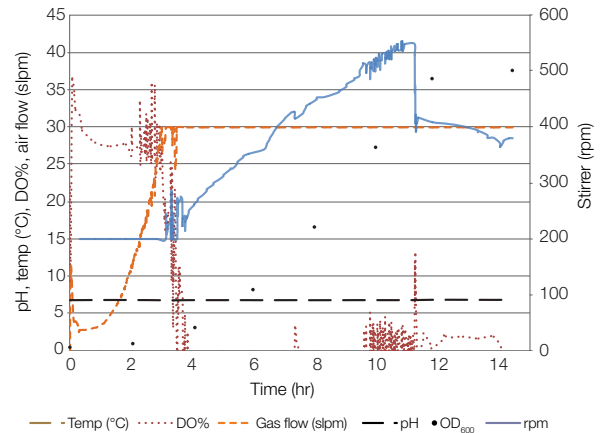


Figure 11. Comparative 7 L *B. subtilis* culture grown in the 10 L stainless steel Sartorius SIP fermentor.

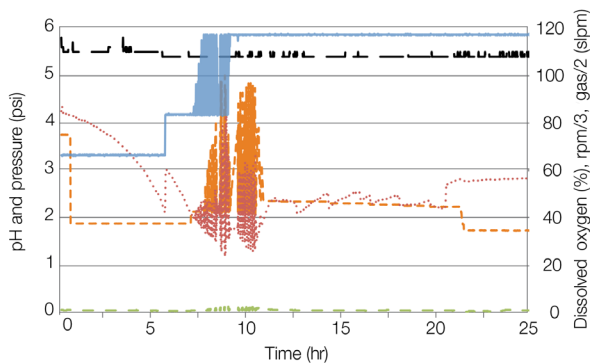


Figure 9. Glucose-fed 300 L *P. pastoris* batch culture.

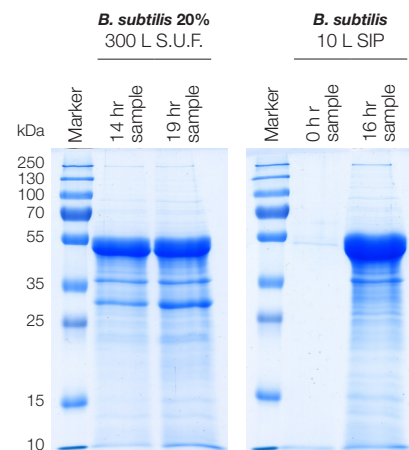


Figure 12. SDS-PAGE of samples from the 300 L S.U.F. *B. subtilis* 20% culture and the 10 L stainless steel fermentor culture.

Conclusion

Growth of an aggressive yeast culture demonstrated the capabilities of the S.U.F. to provide optimal conditions for dense microbial culture growth equal to stainless steel fermentors. The critical parameter set points of the cultures were maintained throughout all the cultures, allowing high-density culture and protein expression requirements to be met. Pressure was maintained below the BPC maximum requirement of 0.5 psi, which confirms that the HyPerforma S.U.F. is able to operate reliably under high gas flow rates.

The single-use condenser system prevented condensate from fouling exhaust filters in all application studies. TCUs for condensers proved adequate for these cultures. Foam was monitored by single-use foam sensors in these culture tests, allowing a single exhaust filter to be utilized during the entire culture. As an option, auxiliary filters are available on custom configurations of the S.U.F. (either to increase the off-gassing capacity or to be available as redundant backup or spare filters).

The S.U.F. is designed to provide sufficient agitation of cultures at volumes down to one fifth of working volume. Our results confirm that mixing, temperature, and mass transfer parameter requirements can be achieved at 20% of maximum rated liquid working volume. This enables the reduction of one seed step, as the final seed can be grown in the S.U.F. and brought to culture volume once reaching desired density. Alternatively, the S.U.F. can be used for scale-up procedures where full working volume is not desired. This S.U.F. vessel can be utilized as a retrofit product to update and replace aging equipment, with the benefit of quick turnaround time and ease of use realized with the latest single-use technologies. We have designed the S.U.F. with an open architecture approach, allowing customers to select the best available technology for a particular application, including proven configurations using Applikon™ or Finesse™ controllers in turnkey fermentors. Ultimately, a completely custom and fully optimized system can be ordered directly from us as a turnkey product that integrates into a full line of supporting single-use products for bioproduction.

Reference

1. *Pichia* Fermentation Process Guidelines, Ver. B. Available at thermofisher.com/pichimanual

Find out more at thermofisher.com/sut

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