Single-Use Fermentors for Plasmid and Minicircle DNA Production

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Demand has surged for good manufacturing practice (GMP)–grade plasmid DNA (pDNA) used in late-phase clinical trials and commercial manufacturing due to the starting material's critical role in producing next-generation therapies. The same is true for *minicircle DNA* (mcDNA) — supercoiled-DNA vectors that can be obtained from plasmids through cutting-edge in vivo recombination techniques. Because mcDNA lacks plasmid backbone sequences and contains primarily active genes, it can provide enhanced transgene expression with greater persistence than conventional plasmids can. Considering the biopharmaceutical industry's great need for both pDNA and mcDNA, tools supporting fast-growing, high-yielding microbial cultures are becoming increasingly important.

Single-use (SU) bioprocess systems offer many advantages over traditional stainless-steel (SS) equipment, including reduced development times from streamlined batch turnover. That accelerates supply of clinical material, helping biotherapeutic manufacturers meet growing demands. SU technologies also minimize the need for equipment cleaning. It is impossible to clean SS fermentors reliably: Although testing of cleaned equipment can detect trace pDNA, no test method can do so reliably for proteins and contaminants of similar sizes. SU culture technologies eliminate many cleaning steps and associated validation activities, saving time and resources while ensuring against contamination.

SU bioreactors have been well studied for applications involving animal cell culture. However, for more technically demanding microbial cultivations, two major barriers have impeded widespread adoption of SU technologies: poor oxygen-transfer capabilities and insufficient heat removal. The Biostat STR Microbial SU fermentation system was designed to overcome those challenges. In collaboration with Sartorius, scientists from PlasmidFactory tested the system's performance a research and development (R&D) facility in Germany. The team evaluated batch and fed-batch culture modes; processes for small and large plasmids, including those used to produce adenoassociated virus (AAV) vectors; and a fermentation protocol adapted for mcDNA.

Because mcDNA is a specialty product that is devoid of plasmid backbone sequences, it represents, from a regulatory perspective, an optimal starting material for production of viral vectors. For example, AAV vector purity has been shown to increase

Figure 1: Trends for critical process parameters (CPPs) during a test fermentation run in a Biostat STR Microbial system (Cum. = cumulative, %sat = percentage of saturation)

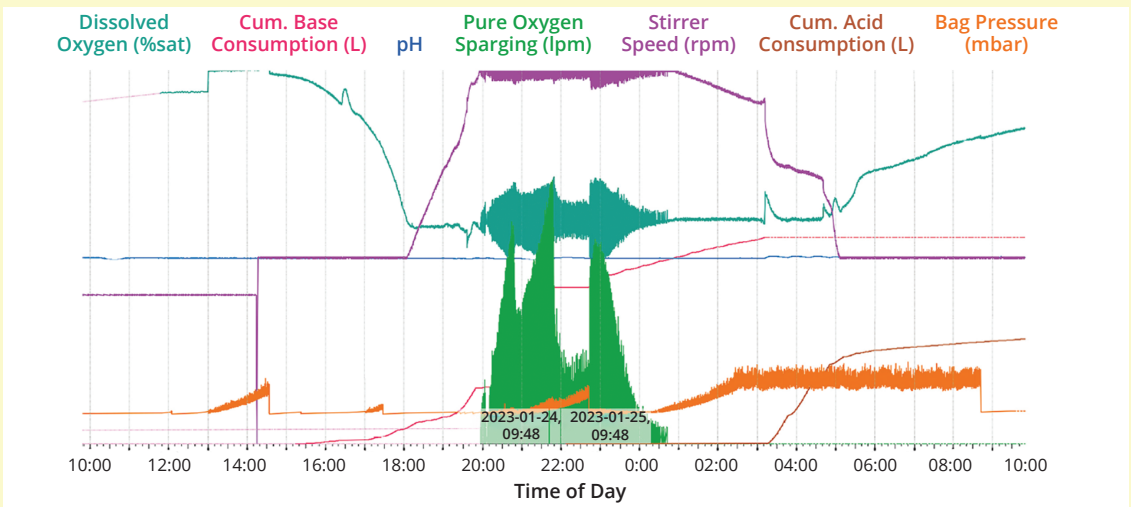


Figure 2: Optical density readings at 600 nm (OD_{600}) during fermentation in a Biostat STR Microbial single-use cultivation system

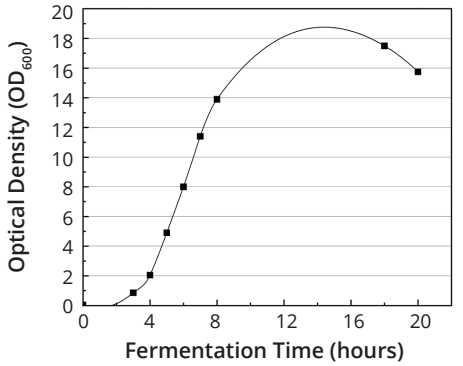
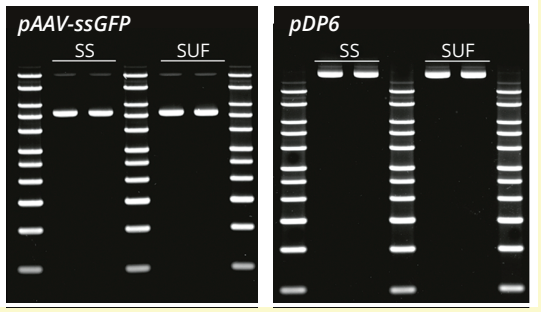


Figure 3: Results from agarose gel electrophoresis of plasmids produced in a stainless-steel (SS) fermentor and a Biostat STR Microbial single-use fermentor (SUF); purified material was for PlasmidFactory's two-plasmid system for production of AAV vectors, including an inverted terminal repeat and reporter gene plasmid (*pAAV-ssGFP*) and a plasmid with helper and packaging sequences (*pDP6*).



significantly when minicircles are used to transfer target genes and helper and packaging constructs: Minicircles decrease the potential for false packaging of bacterial sequences into viral vectors. Additionally, in a final DNA product, removal of extraneous DNA sequences increases the effective dose of the target gene of interest (GoI) on a mass basis.

Materials and Methods

Biostat STR Microbial Fermentation: Initial runs began with sterile pumping of 40 L of proprietary growth medium from PlasmidFactory through one of several available inlets on a Sartorius Flexsafe STR cultivation bag. Using weldable tubes and a BioWelder TC unit ensured sterile connections. Other equipment used included an integrated SU pH electrode, a dissolved oxygen (DO) sensor, and a temperature sensor. An autoclavable, multiuse electrode was connected on line for densitometry measurement. Air-in, air-out, and exhaust-condensate lines were run through aseptic Opta connectors. A Minichiller 600 device (Huber) provided exhaust cooling, and a large, 7-kW process thermostat (Lauda) managed reactor cooling.

AAV Production: Plasmids produced during fermentation were for PlasmidFactory's two-plasmid system for recombinant AAV (rAAV). In one run, the team produced a vector plasmid for an inverted terminal repeat (ITR) and a green fluorescent protein (GFP) reporter gene (*pAAV-ssGFP*).

A second run generated a helper and packaging plasmid for AAV6 (*pDP6*). Samples with 200 ng of DNA underwent electrophoresis in 0.8%, low-melting agarose gel at 7.4 V/cm for 75 minutes. After the run, the gel was stained using GelRed dye (Biotium). Results were measured against a 1-kb DNA ladder molecular-size marker from PlasmidFactory.

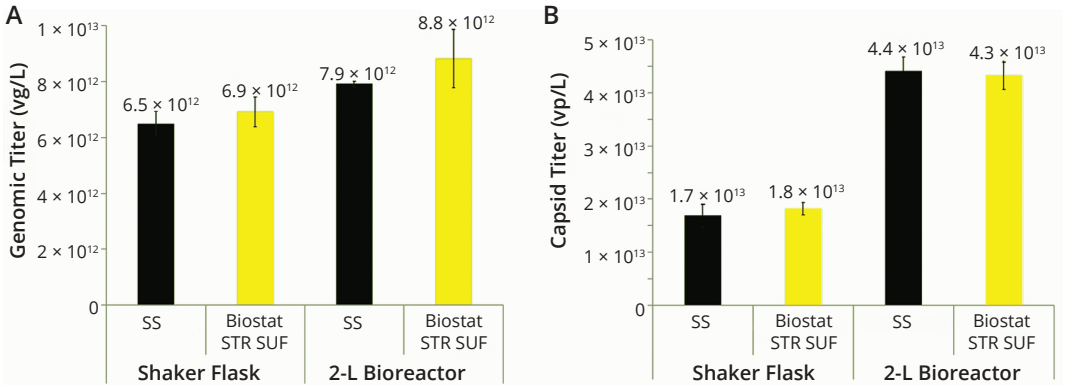
To understand whether pDNA generated in SU fermentors ultimately influenced AAV production, the team evaluated transfection of commercially available human embryonic kidney (HEK293) cells using rAAV6-encoding plasmids produced in either SS fermentors or Biostat STR SU fermentors.

Titer Determination: HEK293 cells cultured in HEK ViP NB media (Sartorius Xell) were transfected at both shake-flask and bioreactor scale using PEI MAX transfection reagent (Polysciences). At 72 hours after transfection, cells were exposed to a detergent-based lysis buffer. Crude lysate from the shake flask underwent centrifugation; bioreactor harvest was clarified by alluvial filtration using a Sartoclear Dynamics Lab kit (Sartorius). Analysts applied a Droplet Digital polymerase chain reaction (ddPCR) assay from Bio-Rad Laboratories to assess genomic titers. Capsid titers were analyzed by enzyme-linked immunosorbent assays (ELISAs) from Thermo Fisher Scientific.

Minicircle Production: A sequence of interest is cloned into PlasmidFactory's proprietary Minicircle production system. Following propagation in an optimized strain, in vivo recombination is triggered, releasing the sequence of interest as a separate molecule. Material is purified using proprietary methods. The result is a pure, supercoiled, monomeric molecule that contains almost exclusively the sequence of interest — e.g., a GoI and associated elements, such as a promoter and polyadenylic acid signal (**1**).

At this stage, the team also tested an on-line sensor for measurement of total cell density (Hamilton Dencytee; data not shown). Those data were collected and correlated with measurements from an off-line spectrophotometer. The goal of incorporating the on-line sensor was to reduce the need for frequent sampling during future processes. The sensor is designed to switch automatically from transmission to reflection at about 1 absorbance unit (AU).

Figure 4: (A) Genomic titers and (B) capsid titers for AAV serotype 6 vectors produced by transfection of human embryonic kidney (HEK293) cells; transfected plasmids were produced in a traditional stainless-steel (SS) fermentor or a Biostat STR Microbial single-use (SU) fermentor. Cultivations were performed at shake-flask and 2-L scale. The values below are means from $N = 2 \pm$ the standard error of the mean (SEM).



Results

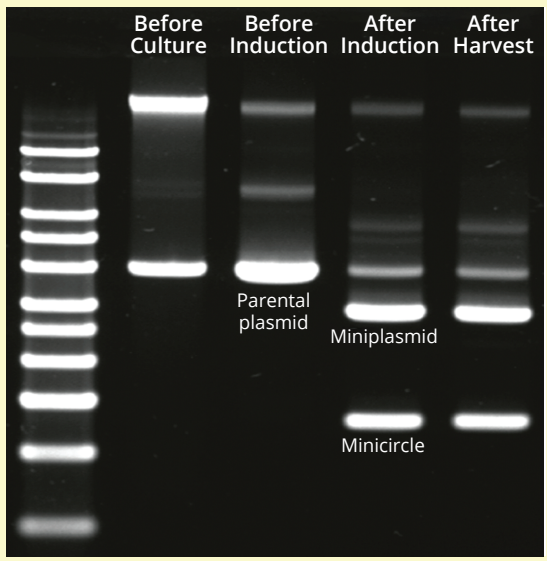
Characterization of Critical Fermentation Parameters

Following recommendations from the Deutsche Gesellschaft für Chemische Technik und Biotechnologie (DECHEMA) expert group on SU technology, the team extensively characterized the Biostat STR Microbial system's performance. They observed a volumetric oxygen mass transfer coefficient (k_La) of up to 675/h, a maximum specific power input of 3 kW/m^3, and a maximum cooling rate of 29 K/h. Those results indicated that the system had sufficient capacity to support efficient transfer of an upstream microbial bioprocess to an SU platform.

An optimized, proprietary production strain of *Escherichia coli* from PlasmidFactory was transformed with the test plasmid and cultivated in a shake flask as the inoculum. After inoculation in the SU fermentor, DO levels dropped gradually, as expected (Figure 1). In addition to cascading the stirrer speed against the DO level, the team set a second level of control through sparging of pure oxygen during maximum stirrer speed (500 rpm). That automatic control was observed for 4 hours during the phase of maximum specific growth rate (Figure 1). Efficient SU exhaust cooling ensured that bag pressure never exceeded 10 mbar, and temperature was kept at 37 °C throughout fermentation (Figure 1). The batch was monitored over 20 hours of cultivation time.

At harvest, analysts noted a final optical density at 600 nm of 15.7 (Figure 2) and a total wet biomass of 1100 g. Analysis of harvested material showed ~1.74 mg pDNA/g of biomass. Thus, the Biostat STR Microbial SU system generated biomass with an acceptable yield of DNA product, and the pDNA quality (homogeneity) was well suited for further downstream processing.

Figure 5: Agarose-gel visualization of recombination during production of minicircle and miniplasmid DNA from a parental plasmid

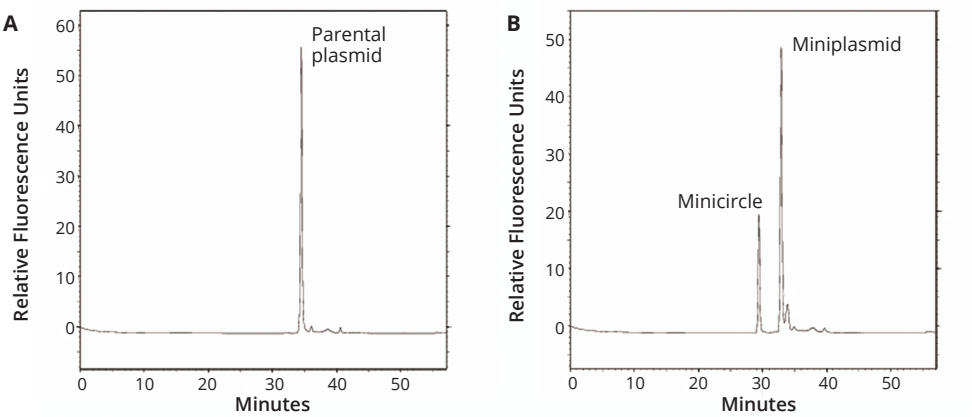


Production of Plasmids for rAAV6

The team produced plasmids for AAV manufacturing based on PlasmidFactory's dual-plasmid system (*pAAV-ssGFP* and *pDP6*). The SS and SU fermentation systems not only achieved similar yields, but also generated plasmids of identical composition, as shown by agarose gel electrophoresis (Figure 3).

After transfection of HEK293 cells using pDNA from an SS fermentor or a Biostat STR Microbial SU system, the team assessed viral titers. Genomic and capsid titers (by ddPCR assay and ELISA, respectively) were highly comparable in shake flasks and 2-L bioreactors (Figure 4). Thus, the SU fermentor generated pDNA that, during transfection, could recapitulate viral titers achieved by pDNA from SS fermentors.

Figure 6: Capillary gel electrophoresis analysis of (A) preinduction and (B) postinduction samples



Production of mcDNA: The team also evaluated mcDNA production in the Biostat STR Microbial SU fermentor, performing two runs. To ensure high yield of mcDNA, it was important to maintain control over the recombination reaction until sufficient amplification of the parental plasmid containing the sequence of interest (Figure 5). Such a process had not been attempted previously in a SU bioreactor, so the resulting data provided novel insights into the feasibility and efficiency of the approach.

The mcDNA fermentation yielded 766 mg of DNA recombination product. To test the reproducibility of the process, production of the same mcDNA was repeated in a subsequent run. That process achieved a far higher specific-DNA productivity, resulting in 914 mg of DNA recombination product after cell lysis, which in turn yielded 518 mg of purified DNA. Based on scale-down purification runs, such amounts are projected to yield pure mcDNA after removal of other DNA molecules at a level representing a higher amount of mcDNA than is needed to start production of chimeric antigen receptor (CAR) T cells based on a nonviral Sleeping Beauty transposition system. In fact, such materials already have entered clinical trials (**2**).

Capillary gel electrophoresis of fermentation material confirmed both effective control of induction and an expected level of recombination to yield mcDNA in a monomeric, supercoiled conformation (Figure 6). Those results show that plasmids produced in the Biostat STR Microbial fermentor retain the desired functional attributes. Thus, the system is a viable alternative to SS fermentors for AAV and mcDNA production platforms.

Conclusion

One critical quality attribute (CQA) for all biomanufacturing processes is the absence of cross-contamination. That factor alone forms a strong case for implementing SU technology. Findings from this study also indicate that SU systems for microbial cultivation no longer require trade-offs among product yield, process safety, and efficiency. SU fermentors now offer real opportunities to transition from traditional SS fermentors to SU operations. For GMP environments, such a shift will be essential in speeding up production and simplifying quality-control activities.

This study was part of ongoing developments at PlasmidFactory, which is building the world's first facility for GMP production of mcDNA.

References

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