

# Fermentation Design for the Manufacture of Therapeutic Plasmid DNA

Aaron E. Carnes

Plasmid DNA production is becoming increasingly important as gene therapies make their way into clinical trials and eventually into the pharmaceutical marketplace. To date, most efforts toward process development have focused on downstream processing. However, the quality of a final product is ultimately determined by fermentation strategy. Through optimization of the growth environment for plasmid-producing organisms, improvements can be achieved in biomass yield, plasmid yield, and plasmid quality.

The primary goal when designing a fermentation process for plasmids is to maximize both the volumetric (mg/L) and specific (mg/g) yields of supercoiled plasmid. Optimizing volumetric yield allows for smaller and more economical fermentations, whereas optimizing specific yield improves plasmid purity in downstream processing. It is also critical that each fermentation produces high-quality plasmids. The FDA recognizes that open-circle, linear, and nicked forms may be less effective therapeutically than supercoiled DNA (1). Those other forms can be very difficult to separate from the

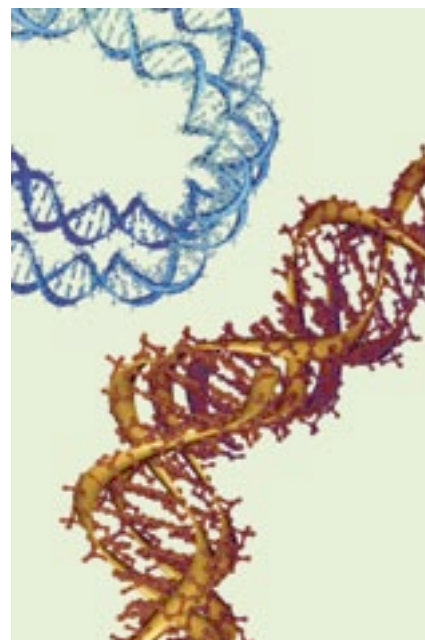
supercoiled plasmid during purification; therefore, fermentation processes should also be optimized to produce a high percentage of supercoiled plasmid.

## PLASMID OPTIMIZATION

Copy number relies intensively on the origin of replication. Most therapeutic plasmids use origins of replication derived from ColE1 or pMB1. Higher yields are possible, in particular, with pMB1-derived pBR322 with a G-to-A mutation (2) or the truncated origin in pUC-derived plasmids than with alternative origins (e.g., pBR322, p15A). Especially useful in fermentation are the temperature-sensitive origins (e.g., pUC, pMM1, pMM7) (3, 4), which allow a 30- to 40-fold increase in plasmid copy number when temperatures shift from 30 °C to 42 °C.

**Plasmid Stability:** Certain sequences can be harmful to the production of high-quality supercoiled plasmid. For greater stability, repeats and palindrome sequences should be avoided as well as AT-rich regions that can make the plasmid susceptible to nicking endonucleases. Because of the complexity of vector construction, many properties associated with certain sequences can be determined only by experimentation with plasmids in the production *Escherichia coli* strain.

Segregational instability, among other mechanisms, may lead to the appearance of plasmid-free cells. Partitioning is usually unregulated with high copy plasmids; as a result, they are not evenly distributed between daughter cells. The probability ( $\Theta$ ) that a plasmid-free



ANDREI TCHERNOV (WWW.ISTOCKPHOTO.COM)

Equation 1:

$$\Theta = 2^{1-N}$$

daughter cell may arise from a plasmid-containing mother cell in the absence of a partition function is expressed by Equation 1, where N is the number of plasmids in the mother cell. That seems to suggest that high-copy plasmids will show little segregational instability, but in practice, plasmid-free cells do arise. It has been suggested that the formation of multimers in a cell reduces the apparent copy number, leading to higher instability. One analysis of multimerization on segregational instability (5) found that if high copy plasmids (copy number >25) show instability, it is from sources other than multimerization.

**PRODUCT FOCUS:** GENE THERAPIES

**PROCESS FOCUS:** PRODUCTION

**WHO SHOULD READ:** PROCESS DEVELOPMENT AND MANUFACTURING

**KEYWORDS:** PLASMIDS, FERMENTATION, MEDIA, *E. COLI*, OPTIMIZATION

**LEVEL:** INTERMEDIATE

Growth-rate-dependent instability is the most likely cause. No matter the reason, high-copy plasmids can impose a metabolic burden on their hosts, giving plasmid-free cells an advantage in culture. Consequently, even a very small number of plasmid-free cells can quickly overtake an entire fermentation.

**Selectable Markers:** Plasmid-free cells can be controlled by using selectable markers in the plasmid. Antibiotic resistance selection is the most common form of selectable markers. There are some regulatory concerns, however. The FDA has stated that ampicillin and other  $\beta$ -lactam antibiotics must not be used because of a risk of hypersensitivity reactions in some patients. To avoid the risk of spreading antibiotic resistance traits to environmental microbes, the agency also discourages use of antibiotic selection for those antibiotics in significant clinical use (6). Currently, kanamycin is most commonly used for antibiotic selection in gene therapy plasmid producers. Concerns about antibiotic resistance have spurred development of alternative selection mechanisms such as balanced lethals and repressor titration (7).

### HOST STRAIN

Plasmid production should be in an *E. coli* K12 attenuated strain considered “automatically exempt” by NIH because it is nonpathogenic and has limited survival if released to the environment. Popular hosts such as DH5 derivatives and XL1 Blue are suitable for plasmid production. Important genetic markers to look for in a host strain include *endA1*, *recA*, and *relA*. The *endA1* mutation is recommended to prevent plasmid degradation after cell lysis. Strains with the *recA* mutation are recombination deficient, ensuring insert stability. And *relA* strains have a relaxed starvation response that may improve the plasmid yield. Both XL1 Blue and DH5 $\alpha$  contain these mutations.

The choice of host strain may also affect downstream purification. For example, HB101 and other carbohydrate producers should be avoided because they can foul downstream processing operations. In minimal media, *gal+* strains produce a large amount of capsule (colanic acid), which can affect harvest

and lysis characteristics. Moreover, many laboratory strains may have unintentional and/or undiscovered mutations due to their years spent in study, so it may be advantageous to try out several strains in hopes of determining an optimal plasmid/host combination.

### MEDIA

Media composition can drastically affect plasmid quality and yield. High-cell-density fermentation requires a balanced medium that supplies adequate amounts of nutrients needed for energy, biomass, and cell maintenance. Until recently, most media optimization efforts have focused on production of recombinant proteins. But media for plasmid production should support high nucleotide pools in cells and supply energy for replication while minimizing other cell activity. The following factors should be considered when formulating media for therapeutic plasmid production:

- Effect of components on plasmid yield and quality
- Biomass yield
- Lot-to-lot consistency
- Potential interference with downstream purification
- Regulatory concerns.

Design of a balanced medium is based on bacterial energy requirements and elemental composition (Table 1). High-cell-density fermentation media commonly contain a carbon source, a nitrogen source, various salts, and trace metals.

Typically, the nutritional requirements are satisfied by either minimal or semidefined media. Minimal media supply only minimum nutritional requirements. A prototrophic strain of *E. coli* can grow on relatively simple media because it can synthesize everything it needs from a simple carbon source, a nitrogen source, and various salts. Fermentation processes using minimal media are highly reproducible. There is also some evidence that minimal media support higher plasmid copy numbers (9).

Semidefined media contain complex components such as yeast extracts, casamino acids, and peptones. Addition of complex components supplies growth factors, amino acids,

**Table 1:** Elemental composition of bacteria (8)

Element	Dry Weight Percentage
Carbon	50–53
Hydrogen	7
Nitrogen	12–15
Phosphorous	2–3
Sulfur	0.2–1.0
Potassium	1.0–4.5
Sodium	0.5–1.0
Calcium	0.01–1.10
Magnesium	0.1–0.5
Chloride	0.5
Iron	0.02–0.20

purines, and pyrimidines — and often supports higher cell densities. A subsequent decrease in reproducibility is not necessarily a problem for plasmid production; however, reliable sources should be used to prevent too much variability over time.

**Carbon Source:** A carbon source provides energy and biomass and is usually the limiting nutrient in fed-batch cultures. Glucose is the conventional choice: It is inexpensive and metabolized very efficiently. However, high glucose levels are known to cause undesirable acetate production due to metabolic overflow (known as the “Crabtree effect”). Glycerol is also used — often as the preferred carbon source in batch cultures. Although cellular yields ( $Y_{X/Glycerol} \approx 0.4$  g/g) are slightly lower than with glucose ( $Y_{X/Glucose} \approx 0.5$  g/g), glycerol does not cause as much acetate excretion, so it can be used at higher concentrations without becoming inhibitory. In semidefined media, carbon from complex components may also be used by cells, so a diauxic growth profile is often observed.

**Nitrogen Source:** The bacterial requirement for nitrogen can be satisfied by inorganic or organic sources. Ammonia and ammonium salts — e.g.,  $\text{NH}_4\text{Cl}$  or  $(\text{NH}_4)_2\text{SO}_4$  — are used in minimal media. Semidefined media supply nitrogen either partly or entirely from complex components: yeast extracts, peptones, and/or casamino acids.

**Salts and Minerals:** Minerals are necessary for bacterial growth, metabolism, and enzymatic reactions. Magnesium, phosphorus, potassium, and sulfur are typically added as distinct media components. Di- and monopotassium phosphates provide potassium and phosphorous and also

## Equations 2–8:

Eq. 2

$$r_p(p, h) = r_p(p) r_p(h)$$

Eq. 3

$$r_p(p) = \frac{V_{pmax} p}{K_p + p}$$

Eq. 4

$$r_p(h) = \frac{V_{hmax} \mu}{K_h + \mu}$$

Eq. 5

$$r_p(p, h) = \frac{V_{max} p \mu}{(K_p + p)(K_h + \mu)}$$

Eq. 6

$$\frac{dp}{dt} = r_p(p, h) - \mu p$$

Eq. 7

$$\frac{V_{max} p \mu}{(K_p + p)(K_h + \mu)} = \mu p$$

Eq. 8

$$p = \frac{V_{max}}{K_h + \mu} - K_p$$

function as buffering agents in certain proportions. Magnesium sulfate heptahydrate is often the source of both magnesium and sulfur. Schmidt et al. (10) have found that high concentrations (~80 mM) are beneficial to production of homogeneous supercoiled plasmid monomers. Other essential minerals include calcium, copper, cobalt, iron, manganese, molybdenum, and zinc. They are required in smaller amounts and often supplied by adding a trace-minerals solution, even though they are usually present as impurities in most major ingredients. Osmolarity is adjusted with sodium chloride.

Use of animal-derived products — in particular bovine products — in plasmid manufacture is unacceptable because of the risk of prion or virus contamination. All media components should be certified as animal product free. Vegetable-derived substitutes are available for many components traditionally of animal origin (e.g., vegetable glycerol and soy peptone).

### PROCESS OPTIMIZATION

One fundamental fact of bioprocessing is that the purity of the final product is proportional to the purity of the starting material. Thus, as the initial fermentation yield of plasmid DNA per gram of bacteria increases, final product purity does as well. Although

high cell densities are important to maximize volumetric yields from culture, increases in the specific plasmid yield also lead to higher purity.

**Growth Rate:** Use of reduced growth rates is a unifying principle in high-quality, high-yield fermentations for plasmid production. High growth rates are associated with acetate production, plasmid instability, and lower percentages of supercoiled plasmid overall. A reduced growth rate alleviates growth-rate-dependent plasmid instability by providing time for plasmid replication to synchronize with cell division.

Many studies have addressed the effect of growth rate on plasmid replication (11, 12). Replication is growth associated: The rate of plasmid replication is affected by the specific growth rate of the cells. The following generalized model of plasmid replication (13) illustrates the relationship of specific growth rate and copy number.

Plasmid replication is assumed to be regulated by two factors: the host cell and the plasmid itself. For the reaction  $p \rightarrow 2p$ , a rate expression for plasmid replication can be written as in Equation 2, where  $r_p(p)$  and  $r_p(h)$  are the plasmid and host cell regulated reaction rates. Because plasmid replication is enzyme regulated, it is expected to follow Michaelis-Menton type kinetics (Equation 3). There,  $p$  is the plasmid number,  $V_{pmax}$  is the maximum rate of plasmid synthesis, and  $K_p$  is saturation constant. A similar expression can be written for the host cell regulated rate (Equation 4). And the two rates are combined to give an expression for  $r_p(p, h)$ , as shown in Equation 5, where  $v_{max} = (V_{pmax})(V_{hmax})$ . A material balance around the cell gives us Equation 6. During balanced growth (exponential phase or continuous culture), the plasmid copy number is constant and  $dp/dt = 0$  (Equation 7).

Solving for  $p$  gives an expression for copy number (Equation 8), which shows that copy number has an inverse relationship with specific growth rate. This unstructured model will not apply in every situation. However, like Monod's unstructured model for microbial growth, it does show the general behavior of plasmid replication during balanced growth.

**Growth Conditions:** Supercoiling is known to be affected by oxygen and temperature (14, 15). Oxygen also has been shown to play a role in plasmid stability. One study found that a single drop in dissolved oxygen (DO) concentration to 5% of air saturation caused a rapid loss in plasmid stability (16). Another showed that fluctuations in oxygen input led to plasmid instability (17). Furthermore, the formation of nicked plasmids and multimers can be affected by many parameters: temperature, pH, DO, nutrient concentration, and growth rate (18). The optimal temperature for *E. coli* growth is 37 °C. However, lower temperatures (30–37 °C) can be used in batch fermentation to reduce the maximum specific growth rate. Temperature can also be adjusted to induce selective plasmid amplification (2). Acidic media (pH 6.2–6.8) and growth at 30 °C have been associated with increased production of pBR322 (19).

### BATCH FERMENTATION

The main advantage of batch fermentation is simplicity. All nutrients for cell growth and plasmid production are present at the time of inoculation. Use of a suitable inoculum (1–5% of the culture volume) is recommended to minimize lag time. During the exponential phase of growth, all nutrients are in excess; thus the specific growth rate will be essentially the maximum specific growth rate ( $\mu_{max}$ ) as predicted by Monod kinetics. As discussed above, slower growth rates are desirable for plasmid production. In batch fermentation the growth rate can be reduced only by reducing  $\mu_{max}$ , which has been achieved by lowering temperatures and using glycerol instead of glucose. Batch fermentation at 30 °C with glycerol will typically result in  $\mu_{max} \leq 0.3 \text{ h}^{-1}$ , which is sufficient to prevent deleterious acetate accumulation and growth-rate-associated plasmid instability (20). Glycerol can also be present at much higher concentrations than glucose without inhibition of growth, leading to higher biomass yields. Generally, yields of up to 60 g/L dry cellular weight (DCW) can be obtained through batch fermentation (Figure 1).

Figure 1: Batch fermentation

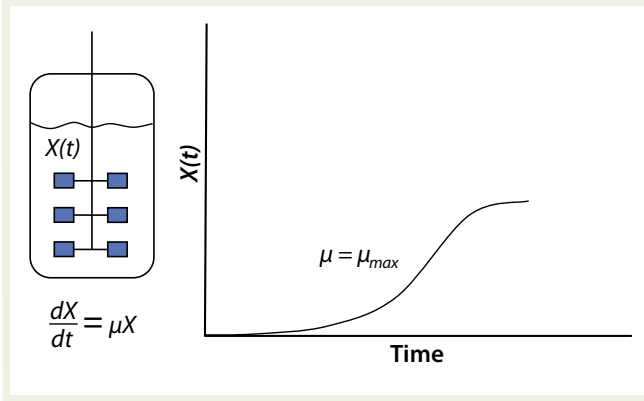
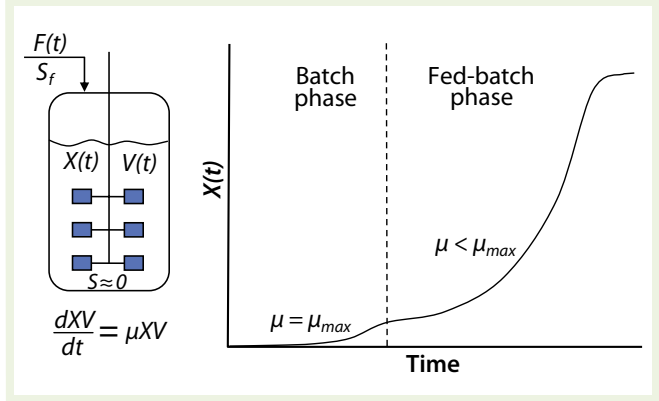


Figure 2: Fed-batch fermentation



### FED-BATCH FERMENTATION

Fed-batch fermentation (Figure 2) is especially useful for plasmid production. Controlled addition of a limiting nutrient allows greater control of growth rates at  $<\mu_{max}$ . Also, fed-batch fermentation provides higher biomass yields than batch fermentation because substrate is supplied at a rate such that it is nearly completely consumed. As a result, conversion of substrate to biomass is very efficient while residual substrate concentration is approximately zero, never reaching inhibitory concentrations. Metabolic overflow from excess substrate is also prevented, reducing the formation of inhibitory acetate.

Fed-batch fermentation starts with a batch phase. Cells are inoculated into an initial volume of medium that contains all nonlimiting nutrients and an initial concentration of the limiting substrate. Controlled feeding of the limiting nutrient begins once the cells have consumed that initial amount. Several feeding strategies have been developed, either feedback controlled (e.g., DO-stat, pH stat, metabolic activity, biomass concentration, and substrate concentration) or predetermined (e.g., constant, linear, stepwise, or exponential feeding).

One of the simplest and most effective feeding strategies is exponential feeding. This method allows a culture to grow at a predetermined rate  $<\mu_{max}$  without the need of feedback control. Such a fermentation begins with a batch mode containing a noninhibitory concentration of substrate. Cells grow at  $\mu_{max}$  until that substrate is exhausted, at which point nutrient feeding begins.

Consider the mass balance equation for substrate concentration,  $S$  (Equation 9), where  $F$  is the nutrient feed rate into the fermentor, and  $S_f$  is the substrate concentration in the feed medium. The term  $FS_f/V$  describes input of substrate, and  $(1/Y_{X/S})\mu X$  describes cellular consumption of substrate. If nutrient is fed to this culture at a rate such that input of substrate is equaled by consumption, a quasi steady state exists for substrate concentration (Equations 10 and 11).

Feeding begins at time  $t_0$ . To calculate the initial feed rate,  $F_0$ , solve for  $F$  at time  $t_0$ . This feed rate increases exponentially according to your desired specific growth rate,  $\mu$  (Equations 12 and 13). The moment at which an initial batch substrate is exhausted usually presents as a sudden rise in DO, or it can be observed by monitoring substrate concentration. Cell concentration may be determined quickly by correlating with optical density (OD) or wet cell weight. Cell concentration at the end of the batch phase also may be estimated from the initial substrate concentration and the yield coefficient,  $Y_{X/S}$ . For example, if the initial concentration of substrate is 30 g/L, and  $Y_{X/S} = 0.5$  g DCW/g substrate, then the cell concentration after all substrate is consumed would be about 15 g/L.

The DO-stat and pH-stat methods are fairly easy to implement because most standard fermentor systems include DO and pH monitoring. Trends in DO and pH can indicate whether substrate is available to the cells. Exhaustion of substrate decreases oxygen uptake, so the DO concentration in the medium rises. The pH will also rise because of an increase in cellular excretion of ammonium ions. Feeding would be

triggered when either DO or pH rises above a set threshold. Growth rate can then be adjusted by changing the threshold value(s).

### EXAMPLES

Lahijani et al. reported a substantial increase in plasmid yield, from 0.03 g to 2.2 g of plasmid in 10-L fermentations using pBR322-derived gene therapy plasmids (2). This improvement was attributed to a temperature-sensitive  $G \rightarrow A$  single-point mutation in the plasmid combined with a temperature shift from 37 °C to 42–45 °C in a fed-batch fermentation.

Schmidt et al. describe their fed-batch process using a glycerol yeast extract medium with DO-stat feedback-controlled feeding (10). The fermentation began with an initial batch volume of 7.5 L. Increased agitation kept DO above 30%. Feed medium was pumped in when DO reached a threshold setpoint of 45%. The culture reached a stationary phase after 41 hours, yielding 60 g/L DCW and 230 mg/L of plasmid (10).

#### Equations 9–13:

Eq. 9

$$\frac{dS}{dt} = \frac{FS_f}{V} - \frac{1}{Y_{X/S}}\mu X$$

Eq. 10

$$\frac{dS}{dt} = 0$$

Eq. 11

$$FS_f = \frac{1}{Y_{X/S}}\mu XV$$

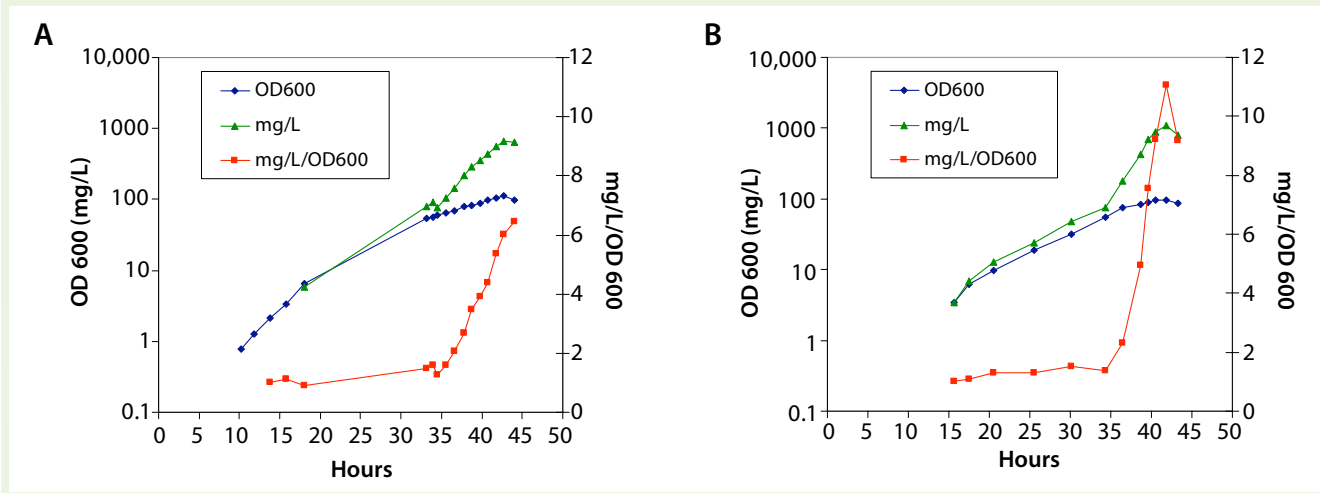
Eq. 12

$$F_0 = \frac{\mu X(t_0) V(t_0)}{S_f Y_{X/S}}$$

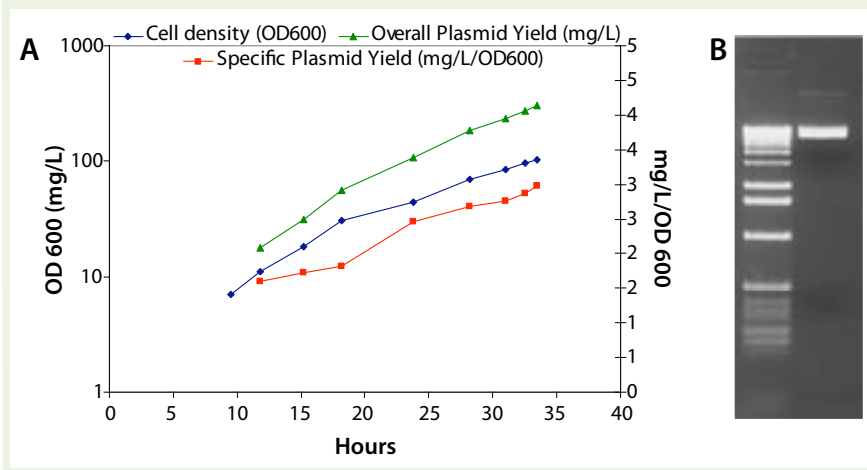
Eq. 13

$$F(t) = F_0 e^{\mu(t-t_0)}$$

**Figure 3:** (A) gWiz-GFP/DH5a fermentations with a 30–37 °C temperature shift at 35 hours; plasmid yield reached 670 mg/L. (B) gWiz-GFP/DH5a fermentations with a 30–42 °C temperature shift at 35 hours; plasmid yield reached 1100 mg/L.



**Figure 4:** (A) Typical growth profile of pBR322-derived plasmids in *E. coli* during fed-batch fermentation with HyperGRO medium. (B) Plasmid DNA produced by the HyperGRO fed-batch fermentation process is highly supercoiled and free of nicked and open isoforms.



Chen et al. used a fed-batch process in semidefined medium with combination DO-stat (threshold setpoint 50%) and pH-stat (setpoint 7.2) feedback control (21). When DO dropped below 30% because of high metabolic activity, agitation speed was increased by a percentage of the previous speed. In a 7-L fermentor, this strategy led to a specific growth rate of 0.13 h<sup>-1</sup> and plasmid yields of 82–98 mg/L (21).

Durland and Eastman report a batch fermentation at 37 °C in a proprietary medium. Their process typically yields 130 mg/L and has yielded as high as 250 mg/L (18).

**Our Experience:** Initial work in our laboratory focused on boosting the fermentation yields of moderate-copy number plasmids (e.g., pBR322-derived: pBR322 replication origin with rop deletion). Our proprietary HyperGRO

balanced medium was designed with amines, salts, vitamins, trace minerals, and a carbon source specifically to favor plasmid production and retention of the supercoiled plasmid form.

Fed-batch fermentations were performed using an exponential feeding strategy. Typically, fed-batch fermentations in HyperGRO media with pBR322-derived plasmids reach a cell density of 100–120 OD<sub>600</sub> or 55–65 g dry cell weight per liter (Figure 3). Plasmid yields average 260 mg/L and have been as high as 430 mg/L (22).

Of particular importance is that our specific plasmid yields (plasmid yield with respect to unit cell mass) are high, typically 2.5–3.8 mg/L/OD<sub>600</sub>, well exceeding levels observed with other fermentation media/processes (4–7). High specific yields are desirable because increasing plasmid yield per

gram of bacteria leads directly to higher final product purities.

#### Experience with High-Copy Gene

**Therapy Plasmids:** Next, we asked whether high-copy plasmids (such as the widely used gene therapy pUC origin plasmids) would give yields as good as or better than the pBR322-derived plasmids in the HyperGRO fed-batch process. We chose the plasmid gWiz-GFP from Gene Therapy Systems of San Diego, CA ([www.genlantis.com](http://www.genlantis.com)) as a well known representative high-copy pUC-origin DNA vaccine plasmid.

Initially, fermentations with gWiz-GFP were not as successful, reaching cell densities of only 15–50 OD<sub>600</sub>. Biomass and plasmid DNA yield data indicate a reduction in specific growth rate and a sharp rise in specific plasmid yield before inhibition of cell growth. The sudden rise in plasmid content to such a high level undeniably placed a metabolic burden on the cell population, which probably caused the reduced growth rate. For temperature-inducible origins such as pUC, growth at low temperature reduces copy number (22).

Based on that information, we modified our fermentation process to use low temperature to reduce plasmid-mediated metabolic burden during biomass production. Fermentation at 30 °C through the growth phase with a shift to 37 °C or 42 °C resulted in productivity yields of 670 mg/L (6.5 mg/L/OD<sub>600</sub>) and 1.1 gm/L (11 mg/L/OD<sub>600</sub>) respectively (23) (Figure 4).

## FUTURE IMPROVEMENTS

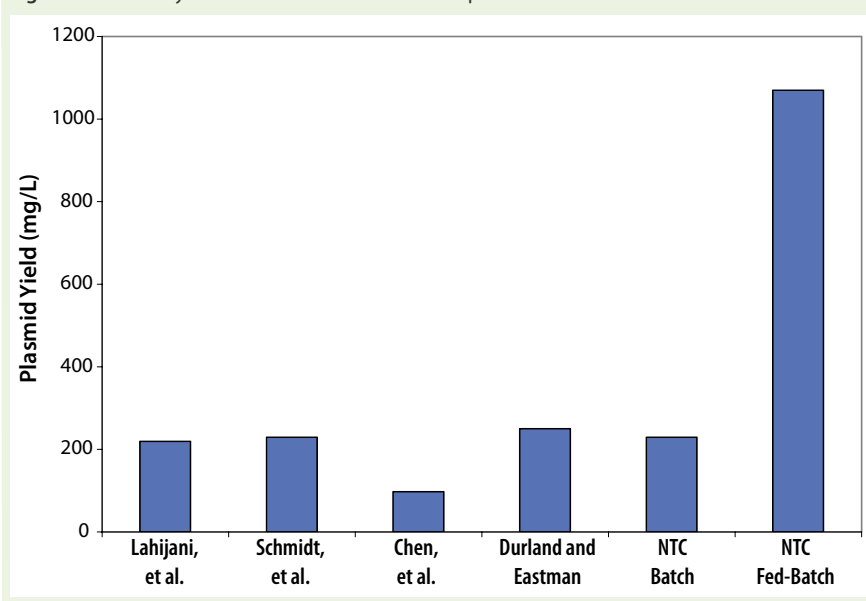
An ever-changing regulatory environment and increasing demand for therapeutic plasmids calls for even greater improvements to ensure product safety and cost-effective processes. High-yield plasmid production in chemically defined minimal media would be a major improvement. Ideally, a host strain could be optimized for growth in minimal media with an increased capacity for plasmid DNA. Preferably that would be non-auxotrophic *gal* to avoid capsule production. Inclusion of DNase, RNase, and protease genes in a strain has been proposed to aid in processing and prevent the use of bovine ribonuclease (24).

**Continuous Culture:** Continuous culture may be desirable for manufacturing very large quantities of plasmid. More can be obtained by increasing productivity rather than increasing volume or the number of fermentations. For example, a typical batch fermentation might last 20 hours and yield 30 g/L DCW. A chemostat culture at steady state with a cell concentration of 30 g/L DCW and a dilution rate of 0.2 h<sup>-1</sup> can produce four times the amount of cell mass in 20 hours at a reduced  $\mu$ . The chemostat also provides a constant environment that may be optimized for plasmid replication. However, some potential problems may arise when using continuous culture (e.g., contamination and mutation due to extended cultivation). Despite those potential problems, the possibilities for much greater productivity call for further investigation of the concept. A detailed discussion of continuous culture is given by Stanbury (8).

## REFERENCES

- 1 Center for Biologics Evaluation and Research. *Points to Consider on Plasmid DNA Vaccines for Preventive Infectious Disease Indications* (Draft Guidance). US Food and Drug Administration: Rockville, MD, December 1996; www.fda.gov/cber/gdlns/plasmid.pdf.
- 2 Lahijani R, et al. High-Yield Production of pBR322-Derived Plasmids Intended for Human Gene Therapy By Employing a Temperature-Controllable Point Mutation. *Hum. Gene Ther.* 7(16) 1996: 1971–1980.
- 3 Wong EM, Muesing MA, Polisky B. Temperature-Sensitive Copy Number Mutants of ColE1 Are Located in an Untranslated Region of the Plasmid Genome. *Proc. Natl. Acad. Sci USA* 79(11) 1982: 3570–3574.

Figure 5: Plasmid yields from several fermentation processes



- 4 Lin-Chao S, Chen WT, Wong TT. High Copy Number of the pUC Plasmid Results from a Rom/Rop-Suppressible Point Mutation in RNA II. *Mol. Microbiol.* 6(22) 1992: 3385–3393.
- 5 Kim BG, Shuler ML. Kinetic Analysis of the Effects of Plasmid Multimerization on Segregational Instability of ColE1 Type Plasmids in *Escherichia coli* B/r. *Biotechnol. Bioeng.* 37(11) 1991: 1076–1086.
- 6 Center for Biologics Evaluation and Research. *Guidance for Human Somatic Cell Therapy and Gene Therapy*. US Food and Drug Administration: Rockville, MD, March 1998: www.fda.gov/cber/gdlns/somgene.pdf.
- 7 Williams SG, et al. Repressor Titration: A Novel System for Selection and Stable Maintenance of Recombinant Plasmids. *Nucleic Acids Res.* 26(9) 1998: 2120–2124.
- 8 Stanbury PF, Whitaker A, Hall SJ. *Principles of Fermentation Technology* (Second Edition). Butterworth-Heinemann: Burlington, MA, USA, 1995.
- 9 O’Kennedy RD, Baldwin C, Keshavarz-Moore E. Effects of Growth Medium Selection on Plasmid DNA Production and Initial Processing Steps. *J. Biotechnol.* 76(2–3) 2000: 175–183.
- 10 Schmidt T, et al. *Method for the Isolation of cc Plasmid DNA*. US Patent 6,664,078 B1 (Qiagen GmbH, Hilden, DE), 16 December 2003.
- 11 Lin-Chao S, Bremer H. Effect of the Bacterial Growth Rate on Replication Control of Plasmid pBR322 in *Escherichia coli*. *Mol. Gen. Genet.* 203(1) 1986: 143–149.
- 12 Shene C, Andrews BA, Asenjo JA. Study of Recombinant Micro-Organism Populations Characterized by Their Plasmid Content Per Cell Using a Segregated Model. *Bioprocess Biosyst. Eng.* 25(6) 2003: 333–340.
- 13 Satyagal VN, Agrawal PA. Generalized Model of Plasmid Replication. *Biotechnol. Bioeng.* 33(9) 1989: 1135–1144.
- 14 Dorman CJ, et al. DNA Supercoiling and the Anaerobic and Growth Phase Regulation of tonB Gene Expression. *J. Bacteriol.* 179, 1988: 2816–2826.
- 15 Goldstein E, Drlica K. Regulation of Bacterial DNA Supercoiling: Plasmid Linking Numbers Vary with Growth Temperature. *Proc. Natl. Acad. Sci. USA* 81(13) 1984: 4046–4050.
- 16 Hopkins DJ, Betenbaugh MJ, Dhurjati P. Effects of Dissolved Oxygen Shock on the Stability of Recombinant *Escherichia coli* Containing Plasmid pKN401. *Biotechnol. Bioeng.* 29(1) 1987: 85–91.
- 17 Namdev PK, et al. Effect of Oxygen Fluctuations on Recombinant *Escherichia coli* Fermentation. *Biotechnol. Bioeng.* 41(6) 1993: 666–670.
- 18 Durland RH, Eastman EM. Manufacturing and Quality Control of Plasmid-Based Gene Expression Systems. *Adv. Drug Deliver. Rev.* 30(1) 1998: 33–48.
- 19 Reinikainen P, et al. *Escherichia coli* Plasmid Production in a Fermenter. *Biotechnol. Bioeng.* 33(4) 1989: 386–393.
- 20 Thatcher DR, et al. *Method of Plasmid DNA Production and Purification*. US Patent 6,503,738 (Cobra Therapeutics, Ltd., Keele, UK) 7 January 2003.
- 21 Chen W. *Automated High-Yield Fermentation of Plasmid DNA in Escherichia coli*. US Patent 5,955,323 (American Home Products Corporation, Madison, NJ) 21 September 1999.
- 22 Carnes A, Williams JA. Optimization of *E. coli* Fermentation for Plasmid DNA Production. *Molec. Ther.* 9(supplement) 2004: 310.
- 23 Carnes AE, Williams J. Process for Plasmid DNA Fermentation. US Patent application PCT/US05/29238, 19 August 2004.
- 24 Carnes AE, Williams J. Improved Strains of *E. coli* for Plasmid DNA Production. PCT/US05/28870, 16 August 2004. 🌐

**Aaron E. Carnes** is director of process development at Nature Technology Corporation, 4701 Innovation Drive, Lincoln, NE 68521; 1-402-472-6530; acarnes@natx.com; www.natx.com.