

Inducible *Escherichia coli* fermentation for increased plasmid DNA production

Aaron E. Carnes¹, Clague P. Hodgson and James A. Williams

Nature Technology Corporation, 4701 Innovation Drive, Lincoln, NE 68521, U.S.A.

Bacterial plasmids are the vectors of choice for DNA vaccines and short-term gene therapeutics. Growing plasmid DNA by microbial (*Escherichia coli*) fermentation is usually combined with alkaline lysis/chromatography methods of purification. To date, typical plasmid fermentation media and processes result in yields of 100–250 mg of plasmid DNA/l of culture medium, using standard high-copy pUC origin-containing plasmids. In order to address this initial and yield-limiting upstream step, we identified novel fermentation control parameters for fed-batch fermentation. The resulting fermentation strategies significantly increased specific plasmid yield with respect to cell mass while enhancing plasmid integrity and maintaining supercoiled DNA content. Fed-batch fermentation yield exceeding 1000 mg of plasmid DNA/l was obtained after reduction of plasmid-mediated metabolic burden during growth, and yields up to 1500 mg of plasmid DNA/l have been achieved with optimized plasmid backbones. Interestingly, by inducing high plasmid levels after sufficient biomass accumulation at low temperature and restricted growth, cells were able to tolerate significantly higher plasmid quantities than cells grown by conventional processes. This 5–10-fold increase in plasmid yield dramatically decreases plasmid manufacturing costs and improves the effectiveness of downstream purification by reducing the fraction of impurities.

Introduction

Escherichia coli plasmids have long been the single most important source of recombinant DNA molecules used by researchers and by industry. At present, plasmid DNA is becoming increasingly important as the next generation of biotechnology products (gene medicines and DNA vaccines) make their way into clinical trials, and eventually into the pharmaceutical marketplace. Plasmid DNA vaccines are finding application as preventive and therapeutic vaccines for viral [1], bacterial [2–4] and parasitic diseases [5,6] and cancer [7–9]. Plasmids are also utilized in gene therapy or gene replacement applications, wherein the desired gene product is expressed from the plasmid after administration to the patient.

As gene therapy and DNA vaccines advance towards approval by the U.S. FDA (Food and Drug Administration, Rockville, MD, U.S.A.), it is essential to devise industrial processes whereby DNA can be economically manufactured not just at the gram scale, but at the kilogram scale and beyond. Increasing the yield (mg of DNA/unit of cell mass) decreases the cost and also increases the purity of the DNA, because it reduces the amount of material being processed. Major impurities in plasmid DNA preparations include: *E. coli* genomic DNA, endotoxin (lipopolysaccharide associated with Gram-negative bacteria), bacterial proteins and RNA. Open-circle (nicked) and linear plasmids result from enzymatic or shear-induced damage to supercoiled plasmid. These damaged plasmid isoforms are currently treated as impurities, since they do not transfect some eukaryotic cells as efficiently as supercoiled plasmid [10]. At present, regulatory standards are not defined, except in preliminary form [9]. However, in the future, international standards for plasmid DNA purity are likely to be the same or very similar to those that are used for recombinant protein products similarly produced from *E. coli* fermentation. Such standards exceed the current purity attainable from established methods. Most glaringly, the FDA guidance published in 1993 [11] recommends the standard of < 100 pg of host genomic DNA per dose (100 pg/1 mg dose is equivalent to one part per ten million or 0.00001%). This level is far below the levels of genomic DNA currently reported for plasmid purification processes (0.01–5%; [12]). Increasing the purity of the starting material, achieving better downstream purification and lowering costs are essential goals for manufacturing clinical grade DNA on an industrial scale.

Therapeutic plasmids typically contain a ColE1- or pBR322-derived replication origin. Common high-copy-number derivatives have mutations affecting copy-number regulation, such as *rop* (repressor of primer gene) deletion [13], and a G → A point mutation [14,15] that can be

Key words: DNA vaccine, *Escherichia coli*, fermentation, gene therapy, plasmid DNA, vector.

Abbreviations used: AAV, adenoviral-associated virus; GFP, green fluorescent protein; LB, Luria-Bertani.

¹ To whom correspondence should be addressed (email acarnes@natx.com).

employed to induce selective plasmid amplification by growth at higher temperature (42 °C) with pUC and pMM1 [16] replication origins.

The use of reduced growth rate coupled with these high-copy replication origins is the unifying principle in high-quality, high-yield plasmid fermentations [17,18]. Generally, lower growth rates favour increased plasmid copy numbers [19,20]. In a study to determine the effects of fermentation strategy on plasmid quality, O'Kennedy et al. [21] found that higher growth rates in batch and fed-batch fermentations were associated with lower percentages of supercoiled plasmid. Lahijani et al. [15] have reported using a pUC origin plasmid in fermentation with exponential feeding and a temperature shift from 37 to 42–45 °C. They achieved a plasmid yield of 218 mg/l. Schmidt et al. [22] describe a fed-batch process using a glycerol/yeast-extract medium with dissolved-oxygen feedback-controlled feeding (DO-stat) producing up to 230 mg/l plasmid. Chen [23] used a fed-batch process in semi-defined medium with a combination of DO-stat and pH-stat feedback control, which led to a specific growth rate of 0.13 h⁻¹ and plasmid yields of 82–98 mg/l. Durland and Eastman [24] report a batch fermentation at 37 °C in a proprietary medium with typical yields of 130 mg/l and as high as 250 mg/l.

Thus existing processes described in the literature reach a plateau at approx. 100–250 mg of plasmid/l. This low yield imposes a cost and purity burden on commercialization of plasmid DNA production processes. We report in the present paper the development of a novel inducible fed-batch fermentation process that breaks this plateau, producing 5–10-fold higher plasmid DNA yield while maintaining plasmid integrity.

Materials and methods

Strains and plasmids

Fermentations were typically performed with *E. coli* strain DH5 α [F- Φ 80*lacZ* Δ M15 Δ (*lacZYA* -*argF*) U169 *recA1* *endA1* *hsdR17*(*r_k*⁻, *m_k*⁺) *phoA* *supE44* λ - *thi-1* *gyrA96* *relA1*], a widely used host for plasmid production. DH5 α includes the *recA* mutation, which minimizes recombination of cloned DNA, and the *endA1* mutation, eliminating non-specific digestion of plasmid.

The pBR322-derived plasmids (plasmids in which the pMB1 replication origin is modified by deletion of the *rop* gene to increase copy number) are of medium copy number and range in size from 5 to 8 kb. Plasmid pW2.0 is a 2.7 kb ampicillin resistance (*amp^r*) pUC19 derivative from our laboratory. Plasmid pmaxGFP (3.5 kb; Amaxa, Cologne, Germany) carries kanamycin resistance (*kan^r*) and has a pUC replication origin. Plasmid gWiz GFP (green fluorescent protein) (Gene Therapy Systems, San Diego, CA, U.S.A.) is a 5757 bp *kan^r* pUC origin DNA vaccine plasmid vector.

Plasmid pNTC7264-hmPA-EGFP (where EGFP is enhanced GFP) is a 6.5 kb *kan^r* pUC-type pDNAVACUltra DNA vaccine plasmid [29] (Nature Technology Corporation, Lincoln, NE, U.S.A.).

Fermentation media

Proprietary semi-defined batch medium (NTC3018) and fed-batch media (NTC3019) were formulated to support high specific plasmid yields, high biomass yields and high plasmid quality. These media are optimized for many components of both batch and fed-batch process media. For example, glycerol is utilized as the carbon source rather than glucose to reduce the maximum specific growth rate during batch fermentation and to minimize acetate production during batch and fed-batch fermentation. Yeast extract is used as a nitrogen source. The trace metals and MgSO₄ concentrations have been optimized on the basis of determined requirements of *E. coli* production strains. Glycerol (50 g/l) and 20 g/l yeast extract, along with salts and trace metals, are used in the batch medium. Fed-batch medium consists of a base medium similar in composition to the batch medium, but with lower concentrations of glycerol (10 g/l) and yeast extract (5 g/l), and a concentrated semi-defined nutrient feed containing 600 g/l glycerol. All components used in the medium are well characterized and certified animal product-free.

Fermentation conditions

The seed cultures were started from glycerol stocks or colonies inoculated into LB (Luria–Bertani) medium plus 50 μ g/ml kanamycin or 100 μ g/ml ampicillin and grown in shake flasks at 37 °C. At mid-exponential phase ($D_{600} = 0.3$ – 1.0), the seed cultures were used to provide 1% inoculum for the fermentations.

All fermentations were carried out in New Brunswick BioFlo 110 bioreactors. During fermentation, pH was controlled at 7.0 ± 0.1 by the automatic addition of 30% ammonium hydroxide or 10% phosphoric acid. The dissolved oxygen probe was calibrated to 0% by nitrogen gas sparging and 100% with air saturation. The vessel was aerated at 1 VVM (volume of gas/volume of medium per min) and dissolved oxygen was maintained at 30% by proportional-integral control of agitation. At cell densities above approx. D_{600} 20, O₂ supplementation was required and was increased automatically as needed during the rest of the fermentation to maintain 30% dissolved oxygen saturation.

During fed-batch fermentations, the semi-defined nutrient feed was added either at a constant feed rate, or according to a predetermined exponential feeding strategy. Briefly, an initial amount of carbon substrate was consumed during the batch phase at a specific growth rate of μ_{max} . Upon exhaustion of the carbon substrate, the fed-batch phase began and the nutrient feed was added continuously.

For exponential feeding, the feed rate was determined automatically by the following equation [17,25]:

$$F(t) = (\mu X_B V_B / S_f Y_{X/S}) \cdot e^{\mu t}$$

where $F(t)$ is the feed rate (litre/h), μ = desired specific growth rate during fed-batch phase (h^{-1}), X_B = biomass concentration at the end of the batch phase (g dry cell weight/l), V_B = initial liquid volume of culture (litre), S_f = limiting substrate concentration in nutrient feed medium (g/l), $Y_{X/S}$ = yield coefficient of biomass from substrate (g/g) and t = time since beginning of fed-batch phase (h).

$Y_{X/S} = 0.4$ g/g, $X_B = 4$ g/l and $\mu = 0.12$ h^{-1} were used for the calculation of feed rate for all exponential fed-batch fermentations and resulted in specific growth rates close to the desired specific growth rate during fed-batch fermentation. Typically, the final culture volume was 20–25% greater than the starting volume due to the addition of nutrient feed and ammonium hydroxide.

Analytical methods

Culture samples were taken at key points and at regular intervals during all fermentations. Samples were analysed immediately for culture density and plasmid yield. Culture density was measured by attenuation (D_{600}) in a Molecular Devices SpectraMAX plus spectrophotometer. Plasmid yield was determined by quantification of plasmid obtained from Qiagen Spin Miniprep kit preparations. Briefly, cells were alkali-lysed and clarified, and plasmid was column-purified and eluted prior to quantification. Cells were diluted in PBS and plated on to LB + kanamycin medium for live-cell determinations. For cell-morphology analysis, 10 μl cell dilutions were incubated for 5 min in the dark with 40 μl of PBS + 10% glycerol containing 1:1000 dilution of SYBR Green I (Sigma, St. Louis, MO, U.S.A.) and observed using fluorescence microscopy. Total DNA analysis was performed either using detergent lysis, as described by Lin-Chao and Bremer [26], or phenol/chloroform lysis, as described by Williams et al. [27]. For phenol/chloroform lysis, 0.5 D_{600} unit of cells was pelleted and resuspended in 200 μl of cell disruption buffer (10 mM Tris/HCl, pH 8.0, 100 mM NaCl and 10 mM EDTA) and disrupted by phenol/chloroform extraction. Isolated plasmid and genomic DNA was resolved on 1% TBE (Tris/borate/EDTA; $1 \times$ TBE = 45 mM Tris/borate/1 mM EDTA) agarose gels and detected by post-staining with a 1:10000 dilution of SYBR Green I. The gels were photographed, scanned as JPEG documents, and genomic and plasmid DNA was quantified using the Kodak ID 3.6 program. The SDS and phenol/chloroform lysis methods gave similar percentage plasmid values. Total protein analysis by SDS/PAGE was performed as described by Williams et al. [27]; 2.0 D_{600} units of cells were pelleted and resuspended in 100 μl of TE buffer (10 mM Tris/HCl,

Table 1 Yields from pUC plasmid batch fermentations with NTC3018 medium

Plasmid	Temperature ($^{\circ}\text{C}$)	Final cell density (D_{600})	Overall plasmid yield (mg/l)	Specific plasmid yield ($\text{mg} \cdot \text{l}^{-1} \cdot D_{600}^{-1}$)
pW2.0	37 shift to 42	57	230	4.0
pmaxGFP	37	16	84	5.3
gWiz GFP	30 shift to 37	54	168	3.12

pH 8.0, and 1mM EDTA), sonicated, mixed 1:1 with $2 \times$ sample buffer [0.125 mM Tris/HCl, pH 6.8, 2 mM EDTA, 6% (w/v) SDS, 20% (v/v) glycerol, 0.025% Bromophenol Blue and 5% (v/v) 2-mercaptoethanol], and resolved on a 4–20%-(w/v)-polyacrylamide/Tris/HCl gel (Bio-Rad, Hercules, CA, U.S.A.).

Results

Effect of fermentation process on plasmid DNA yield

Batch fermentation Batch fermentations were performed with pBR322-derived and pUC-derived plasmids. A pBR322-derived plasmid was grown at 37°C and reached a cell density (D_{600}) of 53 and a plasmid yield of 57 mg/l.

Higher yields were obtained with pUC origin plasmids, pmaxGFP, pW2.0 and gWiz GFP, grown at 37°C or at 30 or 37°C with a shift to 37 or 42°C at mid-exponential phase to induce plasmid-copy-number amplification, as described by Lahijani et al. [15]. The maximum specific growth rate, μ_{max} , of the gWiz GFP culture was 0.26 h^{-1} at 30°C and decreased to 0.11 h^{-1} after the shift to 42°C , likely due to the metabolic burden of increased plasmid replication. Results of these batch fermentations are summarized in Table 1.

Fed-batch fermentation Fed-batch fermentations in NTC3019 media with a variety of medium-copy-number pBR322-derived plasmids were performed. These fermentations were carried out entirely at 37°C because these plasmids are medium copy number and do not contain a temperature-sensitive origin of replication. Multiple fed-batch fermentations with exponential feeding reached cell densities of D_{600} 100–120 (55–65 g dry cell weight/l) with plasmid yield averaging 260 mg/l and reaching as high as 438 mg/l. Importantly, the specific plasmid yields were high, typically between 2.5 and 3.8 $\text{mg} \cdot \text{l}^{-1} \cdot D_{600}^{-1}$, and appear to be growth-associated (Figure 1). These high yields are presumably due to an optimized combination of growth media, host strain and the exponential fed-batch fermentation strategy.

Fed-batch cultivations in NTC3019 media at 37°C with plasmid gWiz GFP and other pUC origin plasmids typically resulted in final cell densities less than D_{600} 20 and poor overall plasmid yields due to the reduced biomass. We

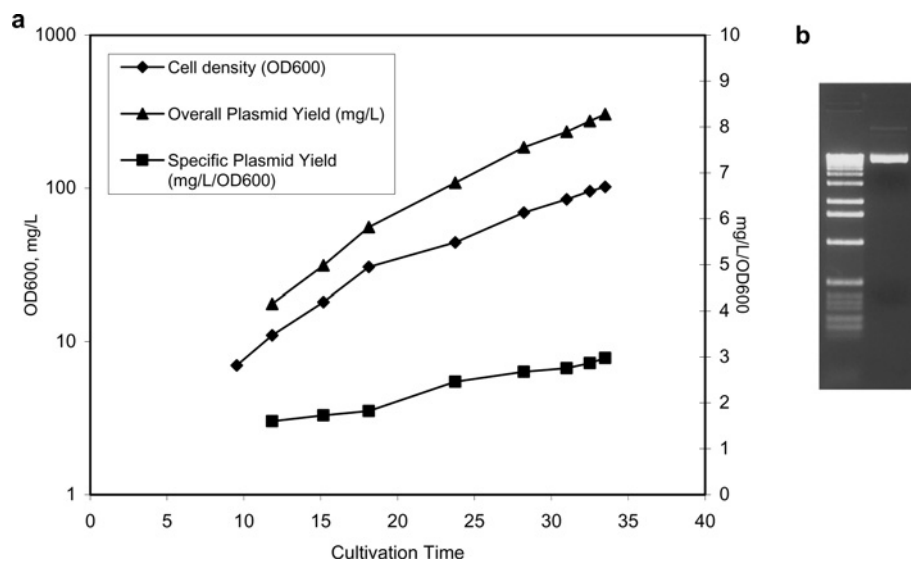


Figure 1 pBR322-derived plasmid fed-batch fermentation in *E. coli* with NTC3019 media

(a) Typical growth profile of pBR322-derived plasmids in *E. coli* during fed-batch fermentation with NTC3019 media; (b) plasmid DNA produced by the NTC3019 media fed-batch fermentation process (right) is highly supercoiled (main band) and free of nicked and open-circle isoforms. The 1 kb DNA ladder is shown (Invitrogen) (left). OD600, attenuation (D_{600}).

suspected that metabolic burden by high plasmid copy numbers may be related to the low biomass yields. pUC origin plasmids are of high copy number and are temperature-sensitive, with copy numbers increasing with temperature.

In an experiment to examine temperature effects, a gWiz GFP fed-batch culture was started at 37°C. The nutrient-limited fed-batch phase began 10 h after inoculation, after which biomass growth slowed and stopped at approx. D_{600} 15 (Figure 2a). Plasmid yield analysis indicated an elevated specific plasmid yield of $2.7 \text{ mg} \cdot \text{l}^{-1} \cdot D_{600}^{-1}$ as the cell growth began to stop. The temperature was then reduced to 33°C in an attempt to reduce the plasmid copy number and thus alleviate the metabolic burden on the cells. The specific plasmid yield decreased to $1.6 \text{ mg} \cdot \text{l}^{-1} \cdot D_{600}^{-1}$ and cell growth resumed; however, the specific plasmid yield gradually rose again and biomass growth stopped at approx. D_{600} 60 instead of growing to $> D_{600}$ 100 as expected, even though feed nutrient was still being added.

Fluorescence microscopy showed extensive filamentation of cells taken as the growth halted at 37°C (Figure 2b), suggesting that inhibition of cell division caused growth arrest, which would have likely eventually led to lysis [28]. After the temperature was reduced to 33°C, a sample of growing cells showed much less filamentation (Figure 2b).

A possible explanation for low final biomass was that the cell population never fully recovered from filamentation and viability loss when begun at 37°C. To test this, two fed-batch fermentations were carried out entirely at 33°C. In both fermentations, the culture reached a peak at cell

densities less than D_{600} 60. Biomass and plasmid DNA yield results from these fermentations indicated 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 places a metabolic burden on the cell population, which may be the cause of the reduced growth rate.

Inducible fed-batch plasmid fermentation The growth-arrest problem was finally overcome by using an initial temperature setpoint of 30°C in order to keep the plasmid copy number at a minimum, thus reducing the metabolic load while accumulating biomass. Temperature shifts to 37 or 42°C were performed at D_{600} 60 to increase the plasmid copy number prior to harvest. Surprisingly, volumetric plasmid yields with gWiz GFP were 670 mg/l when shifted to 37°C, and 1070 mg/l when shifted to 42°C (Figure 3). Specific plasmid yield and biomass increased for at least 9 h following the temperature shifts. This strategy (Figure 4) allowed the cultures to reach higher cell densities, ultimately exceeding D_{600} 100 with no filamentation or loss of cell viability.

Feeding strategy Two different feeding strategies, constant and exponential feeding, were evaluated with a 6.5 kb Nature Technology Corporation pDNAVACCultra [29] plasmid, pNTC7264-hmPA-EGFP, in cultures with initial volumes of 4 litres. Feeding for both strategies started 15 h post-inoculation. A feed rate of 20 ml/h (3.0 g of glycerol $\cdot \text{h}^{-1} \cdot \text{l}^{-1}$) was used for constant feeding; this feed rate was chosen as the highest feed rate that would not result in accumulation of glycerol in the culture. Exponential feeding was carried out as described in the 'Fermentation

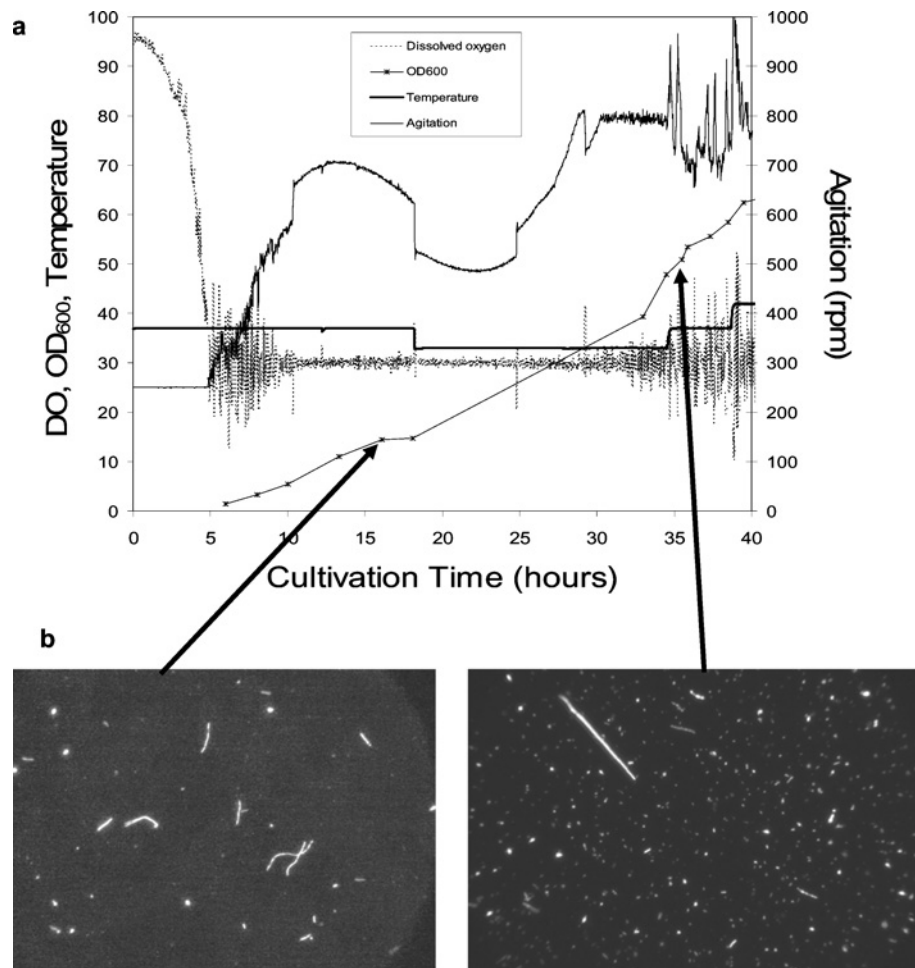


Figure 2 *gWiz* GFP plasmid fed-batch fermentation in *E. coli* with NTC3019 medium

(a) Growth and control parameter profile [dissolved oxygen (DO), culture turbidity ($OD_{600} = D_{600}$), temperature ($^{\circ}\text{C}$) and agitation (rpm = rev./min)] of a fed-batch fermentation profile of *gWiz* GFP; (b) fluorescence microscopy of cells stained with SYBR Green I shows filamentation at the plateau (left), whereas growth resumed and filamentation was decreased after the temperature was reduced to 33°C (right).

conditions' subsection above, with an initial feed rate of 8 ml/h, increasing at an exponential rate of 0.12 h^{-1} . The temperature was shifted from 30 to 42°C at a D_{600} of 39–41 for both fermentations. The constant feeding strategy resulted in a maximum cell density of D_{600} 64, a plasmid yield of 620 mg/l and a specific plasmid yield of $9.73\text{ mg}\cdot\text{l}^{-1}\cdot D_{600}^{-1}$ after 43.8 h. While this yield is still high when compared with previous results with other plasmids, the exponential feeding strategy was clearly better than the constant feed rate tested and resulted in a cell density of D_{600} 85.8 and an extremely high plasmid yield of 1497 mg/l with a specific plasmid yield of $17.5\text{ mg}\cdot\text{l}^{-1}\cdot D_{600}^{-1}$ after only 41.4 h (Figure 5). The final culture volumes were 4.57 and 4.93 litres for the constant and exponential fed-batch fermentations respectively. The advantage of exponential feeding is especially apparent in the difference between plasmid productivity over the duration of

the fermentations; the constant feed fed-batch had a plasmid productivity of $14\text{ mg}\cdot\text{l}^{-1}\cdot\text{h}^{-1}$, while the exponential feed fed-batch had a plasmid productivity of $36\text{ mg}\cdot\text{l}^{-1}\cdot\text{h}^{-1}$.

Plasmid yields prior to the temperature shift remained low ($<2\text{ mg}\cdot\text{l}^{-1}\cdot D_{600}^{-1}$). Remarkably, the specific plasmid yields after temperature shift are very high, up to $17.5\text{ mg}\cdot\text{l}^{-1}\cdot D_{600}^{-1}$, well exceeding published yields (Table 2). Interestingly, after the temperature shift, the cells were able to tolerate significantly higher quantities than growing cells from the 37°C fed-batch process in the same media.

Total DNA and protein analysis

An additional fermentation with a 42°C temperature shift was performed with a 6.4 kb *kan*^r, pUC origin, DNA vaccine plasmid, VRC 5737 from the Vaccine Research Center;

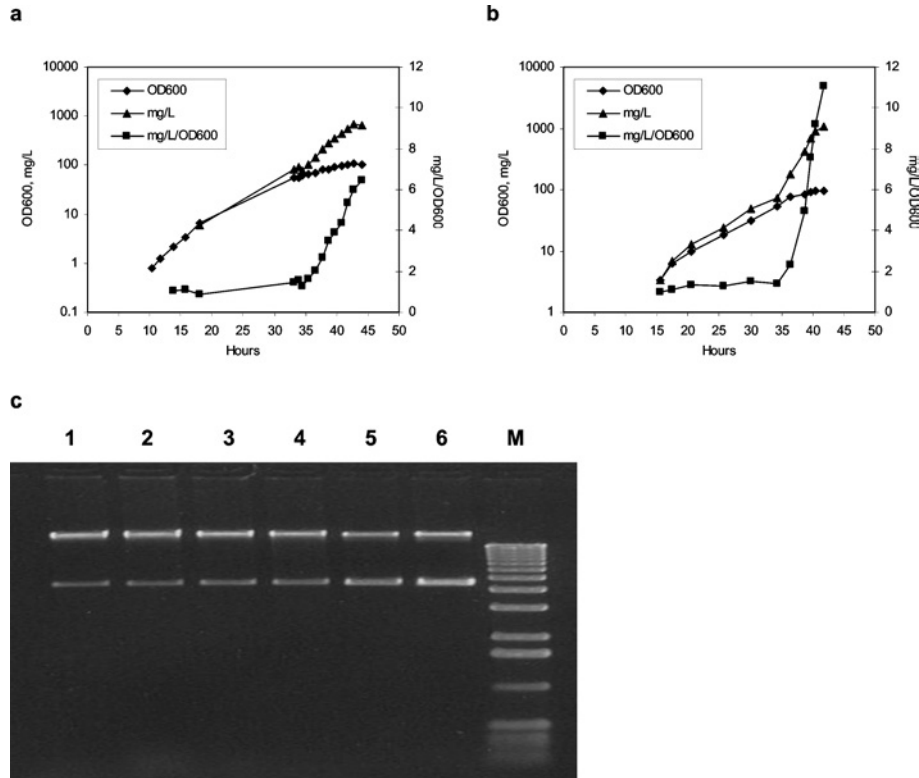


Figure 3 Inducible plasmid production process

gWiz GFP/*E. coli* DH5 α inducible fed-batch fermentation in *E. coli* with NTC3019 medium (37 or 42 °C induction) is shown: (a) growth and plasmid yield profile of a fermentation with a 30 \rightarrow 37 °C temperature shift at 35 h; plasmid yield reached 670 mg/l; (b) growth and plasmid yield profile of a fermentation with a 30 \rightarrow 42 °C temperature shift at 35 h; plasmid yield reached 1100 mg/l; and (c) total DNA analysis from sample time points of a VRC 5737/*E. coli* DH5 α fermentation with a 30 \rightarrow 42 °C temperature shift (see Table 3). The lower band is supercoiled monomer plasmid, the larger band is genomic DNA and supercoiled dimer plasmid. From left to right: total DNA from shake flask inoculum (lane 1), batch phase fermentation, 30 °C, D_{600} ('OD600') 4 (lane 2), fed-batch phase fermentation, 30 °C, D_{600} 18 (lane 3), fed-batch phase fermentation, 30 °C, D_{600} 45 (lane 4), fed-batch phase fermentation, 30 °C, $T = 4$ h post 42 °C shift, D_{600} 70 (lane 5), and fed-batch phase fermentation, $T = 6$ h post 42 °C D_{600} 83 (lane 6). Lane M, the 1 kb DNA ladder (Invitrogen).

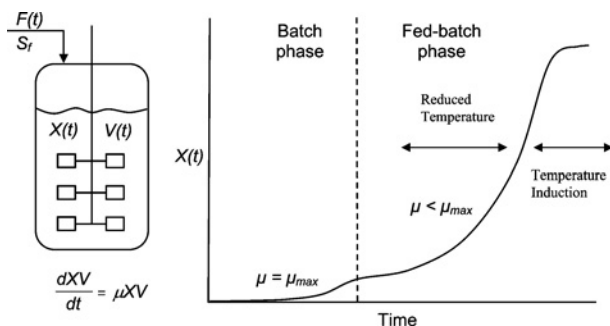


Figure 4 Inducible fed-batch fermentation process

Bethesda, MD, U.S.A., to determine yields with an alternative plasmid, as well as to monitor plasmid DNA quality during the process. Induction was performed by shifting from 30 to 42 °C at a D_{600} 53. Samples from throughout the fermentation were analysed for plasmid DNA, total DNA and total protein. The results are summarized in Table 3, and

the total DNA profile from the samples is shown in Figure 3(c). The yield and purity were similar to those obtained with plasmid gWiz GFP. Plasmid DNA production was dramatically induced after the 42 °C temperature shift, with a specific yield of $9.1 \text{ mg} \cdot \text{l}^{-1} \cdot D_{600}^{-1}$ and overall yield of 751 mg/l 6 h post-induction. Consistent with this, analysis of total DNA from samples throughout the fermentation demonstrated dramatic enhancement in the ratio of plasmid to genomic DNA after induction (plasmid content increased from 22% total DNA in the shake culture inoculum to 61% total DNA at $T = 6$ h post 42 °C induction). This represents an enrichment of plasmid content from 0.3 to 1.6 times genomic DNA. Importantly, the fraction of monomeric plasmid was stable throughout the fermentation, and nicked or linearized versions of the plasmid were undetectable (Figure 3c). Such low levels of nicked plasmid is unusual for plasmid fermentation processes, and may reflect possible enhanced DNA compaction and packaging that both increases cell carrying capacity and protects DNA from endonuclease digestion (see the Discussion).

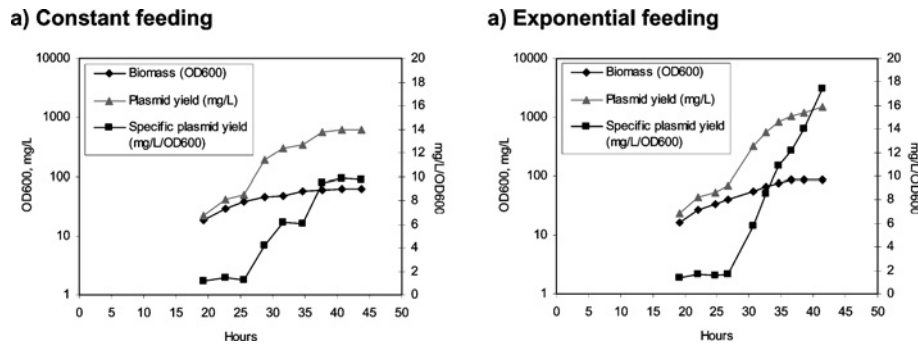


Figure 5 Inducible fed-batch plasmid fermentation with pNTC7264-hmPA-EGFP/*E. coli* DH5 α with temperature shift from 30 to 42 °C at D_{600} 39-41 using constant or exponential nutrient feeding

(a) Constant nutrient feed rate: plasmid yield reached 620 mg/l, $9.73 \text{ mg} \cdot \text{l}^{-1} \cdot D_{600}^{-1}$ at D_{600} ('OD600') 64. (b) Exponential nutrient feed rate: plasmid yield reached 1497 mg/l, $17.5 \text{ mg} \cdot \text{l}^{-1} \cdot D_{600}^{-1}$ at D_{600} 85.8.

Table 2 Comparison of plasmid yield and productivity of various fermentation processes

Fermentation process	Plasmid	Cell density (D_{600})	Volumetric plasmid yield (mg/l)	Specific plasmid yield ($\text{mg} \cdot \text{l}^{-1} \cdot D_{600}^{-1}$) ^a	Plasmid productivity ($\text{mg} \cdot \text{l}^{-1} \cdot \text{h}^{-1}$) ^b
Schmidt et al. [22] (fed-batch)	pUK21CMV β (pUC-type origin)	140	200	1.4	5.6
Lahijani et al. [15] (fed-batch)	VCL1005G/A (pUC-type origin)	78	218.6	2.8	11
The present study (NTC3018, batch)	pW2.0 (pUC origin)	57	230	4.0	16
The present study (NTC3019, 37 °C, fed-batch)	pBR322-derived	121	438	3.6	13
The present study (NTC3019 30 \rightarrow 42 °C, inducible fed-batch)	gWiz GFP (pUC-type origin)	97	1070	11	26
The present study (NTC3019 30 \rightarrow 42 °C, inducible fed-batch)	pNTC7264-hmPA-EGFP (pUC-type origin)	85.8	1497	17.5	36

^a Expressing plasmid yields in terms of specific yield ($\text{mg} \cdot \text{l}^{-1} \cdot D_{600}^{-1}$) indicates the amount of plasmid relative to the total cell mass.

^b Productivity was determined by dividing the volumetric plasmid yield by fermentation time.

Table 3 Plasmid production kinetics

Sample	D_{600}	Plasmid yield (mg/l)	Plasmid yield ($\text{mg} \cdot \text{l}^{-1} \cdot D_{600}^{-1}$)	Plasmid purity ^a (% total DNA) ^b	Plasmid quality ^a (% supercoiled monomer)
Flask (37 °C)	0.8	–	–	22	–
Batch (30 °C)	4	5	1.4	24 (26)	91
Fed-batch 30 °C	18	24	1.3	30 (33)	91
	45	77	1.7	33 (36)	92
42 °C	70	337	4.8	51 (55)	93
	83	751	9.1	57 (61)	93

^a Undetectable levels of nicked or linearized plasmid in all purified plasmid and total DNA samples (see Figure 3c).

^b Percentage supercoiled monomer only; the supercoiled dimer plasmid co-migrates with genomic DNA. Values in parentheses are estimated total supercoiled plasmid, based on percentage supercoiled monomer.

Table 4 Yields from vaccine plasmid fed-batch fermentations with NTC3019 medium

Plasmid name	Target antigen	Induction D_{600}	Final D_{600}	Overall yield (mg/l)	Specific yield ($\text{mg} \cdot \text{l}^{-1} \cdot D_{600}^{-1}$)
VRC 4314	HIV gag	55	86	540	6.3
VRC 4401	HIV gag	30	56	577	10.3
VRC 4403	HIV pol	57	74	426	5.8
VRC 4404	HIV nef	26	54	377	7
VRC 5736	HIV Clade A envelope	26	66	334	5.1
VRC 5737	HIV Clade B envelope	55	97	820	8.5
VRC 5738	HIV Clade C envelope	27	62	606	9.8
VRC 5738	HIV Clade C envelope	30	67	733	11

SDS/PAGE analysis of total protein preparations from the samples detected high levels of a 31 kDa protein in all samples; this protein was induced approx. 2-fold after 6 h induction at 42 °C. This protein correlates with the expected size and abundance of the plasmid-borne kan^r gene product [30]. Additional proteins of approx. 60 and 85 kDa were induced during fed-batch growth at 42 °C, or during the entire fed-batch phase (30 and 42 °C) respectively. No other alterations in protein expression were observed (results not shown).

Process robustness

The inducible process was performed utilizing a series of seven VRC pUC origin-containing DNA vaccine plasmids each containing different target antigens (Table 4). The effect on plasmid yield of inducing plasmid production by shifting to 42 °C at cell densities of either D_{600} 25–30 or 55–60 was determined. The results (Table 4) demonstrate consistently high yield when the induction is between 55 and 60 D_{600} (five out of seven fermentations > 500 mg/l plasmid). Three out of five fermentations with induction at D_{600} 25–30 also exceeded 500 mg/l plasmid. The overall plasmid yield at

harvest is lower when induced at 25–30 D_{600} (average yield is 525 mg/l versus average yield of 625 mg/l when induced at D_{600} 55–60) due to reduced overall biomass. The specific plasmid yield after induction was high when induced at either 25–30 or 55–60 D_{600} .

Multiple other plasmids with various pUC origin backbones, including different antibiotic-resistance genes and orientations of prokaryotic elements, have been produced in yields greater than 500 mg/l in NTC3019 media, using the 30–42 °C inducible process in the *E. coli* DH5 α or *E. coli* DH1 strains (results not shown). Collectively, these results demonstrate that the inducible process is not specific to a particular *E. coli* strain or a specific plasmid.

Process consistency

The consistency of the inducible process was evaluated utilizing a single VRC DNA vaccine plasmid (VRC 5737) in ten 10 litre fermentation runs, all induced between D_{600} 50 and 55. The results (Table 5) demonstrate high process consistency. No significant differences in specific plasmid yield were observed between 6 and 8 h of induction. Total DNA analysis of harvest samples from fermentations 8 and 9 demonstrated that 61 % of the total cellular DNA was supercoiled monomer plasmid.

Discussion

A fed-batch fermentation process was developed in which plasmid-containing *E. coli* cells were grown at a reduced temperature during the fed-batch phase, during which growth rate was also restricted. This was followed by a temperature up-shift and continued growth at elevated temperature to accumulate plasmid (Figure 4). This method, which achieves high specific plasmid yields and high cell densities, unexpectedly and dramatically improves the final volumetric and specific yield of pUC origin plasmids while maintaining plasmid integrity. The plasmid DNA produced with the process is of high quality, being essentially 100 % supercoiled

Table 5 Data from ten 10 litre fed-batch fermentations with plasmid VRC 5737 showing process consistency

Run no.	Induction D_{600}	Final D_{600}	Overall plasmid yield (mg/l)	Specific plasmid yield ($\text{mg} \cdot \text{l}^{-1} \cdot D_{600}^{-1}$)	Temperature shift (h)	Begin harvest (h)	Time at 42 °C (h)
1	54	97	820	8.5	31:38	38:45	7:07
2	51	92	751	8.2	31:24	37:40	6:16
3	50	93	783	8.4	31:50	38:27	6:37
4	51	90	851	9.4	32:15	40:16	8:01
5	53	93	829	8.6	32:49	39:45	6:56
6	53	83	737	8.9	31:59	39:16	7:17
7	53	73	814	11.2	30:43	38:11	7:28
8	55	82	814	9.9	32:47	39:33	6:46
9	51	91	848	9.3	32:22	39:20	6:58
10	53	89	928	10.5	31:06	38:16	7:10

with no detectable nicking, deletion or other rearrangement (Figure 3c). The method is simple, can be used with multiple pUC-based backbones and does not require prescreening of individual colonies for high producing cell lines. While yields up to 1.5 g of plasmid/l are reported with this method, we speculate that the total plasmid yields are actually higher. The analytical methodology employed herein determines the amount of plasmid that can be purified by standard alkaline lysis and column downstream processing, and is not a total plasmid assay, such as an HPLC assay on total cell lysates.

The exponential feeding strategy is preferred over constant feeding. Exponential feeding is simple, effective and results in a nearly constant predetermined specific growth rate less than the maximum specific growth rate without the need of feedback control. Furthermore, exponential feeding resulted in higher plasmid productivity, volumetric yield and specific yield than constant feeding at the feed rate tested.

The inducible fed-batch process described herein maintained low ($< 2 \text{ mg} \cdot \text{l}^{-1} \cdot D_{600}^{-1}$) plasmid levels during the growth phase of the process before the temperature shift, and facilitated unprecedented ultra-high plasmid production ($6\text{--}17.5 \text{ mg} \cdot \text{l}^{-1} \cdot D_{600}^{-1}$) after the temperature shift. Expressing plasmid yields in terms of specific yields ($\text{mg} \cdot \text{l}^{-1} \cdot D_{600}^{-1}$) indicate the amount of plasmid per unit cell mass. High specific yields are very desirable, since increased plasmid yield per gram of bacteria leads directly to higher final product purities. At a specific yield of $9.1 \text{ mg} \cdot \text{l}^{-1} \cdot D_{600}^{-1}$, plasmid DNA accounted for 61 % of total DNA or 1.6 times genomic DNA (Table 3), and it can be reasonably assumed that the ratio of plasmid to genomic DNA is even higher at a specific plasmid yield of $17.5 \text{ mg} \cdot \text{l}^{-1} \cdot D_{600}^{-1}$. This greatly reduces the amount of genomic DNA that needs to be eliminated during downstream processing. Consistent with this, gram-scale plasmid purifications utilizing cells from these processes have attained excellent yields of highly pure supercoiled plasmid, with greatly reduced levels of residual genomic DNA (A. E. Carnes and J. A. Williams, unpublished work).

O'Kennedy et al. [31] have shown that the fermentation process is a critical factor not only for plasmid yield and quality, but also for the effectiveness of downstream purification, with the initial lysis steps being specifically sensitive to the fermentation process. Cells from this fermentation process have shown no difficulties with the alkaline lysis methods, such as the standard alkaline lysis or a modified alkaline lysis [32], widely used for initial large-scale plasmid recovery. Although this fermentation process produces highly supercoiled plasmid, free of open-circle and linear isoforms, as with other processes, care must still be taken during downstream purification to prevent damage to the supercoiled plasmid. Plasmid is most susceptible to damage during the denaturation step of alkaline lysis; Clemson and

Kelly [33] describe the optimization of alkaline lysis for maximum recovery and percentage supercoiled plasmid.

Production of unstable plasmids

A key advantage of the inducible fed-batch process is that maintaining low plasmid levels during growth allows: (i) fermentation with high producing cell lines that would otherwise be toxic due to plasmid-mediated metabolic burden; and (ii) production of plasmids containing unstable sequences. Palindromic sequences, direct or inverted repeats, and Z-DNA-forming sequences are often deleted or rearranged by *E. coli* hosts. Some plasmids for therapeutic use must contain unstable sequences [e.g. inverted or direct repeats for shRNA (short-hairpin RNA) therapeutics and for viral vectors such as AAV (adenoviral-associated virus) and HIV] and are prone to dimerization. Several host strains are available for propagation of unstable plasmids, for example, Sure cells (Stratagene), GT116 (Invivogen) or Stbl2 (Invitrogen, Carlsbad, CA, U.S.A.). Stabilization is maximal at low temperature (i.e. 30°C), presumably by plasmid-copy-number reduction. This strategy obviously increases production cost because of the decreased plasmid yield. Use of the inducible fermentation process described here allows propagation at 30°C of unstable plasmids in stabilizing cell lines, prior to increasing copy number only for a short duration prior to harvest. Amplification of plasmid copy number after suitable biomass accumulation should help preserve quality and stabilize toxic plasmids, while maximizing yield. This is because selection pressure at the cellular level is reduced during the biomass accumulation phase by minimizing the growth rate difference between monomer or dimer plasmid-bearing and plasmid-free cells. This is demonstrated by the observed stability of a plasmid with respect to multimerization during the process (Table 3).

We have used this process to successfully produce long-terminal-repeat-containing plasmids, as well as deletion-prone AAV helper plasmids, in high yield ($> 500 \text{ mg/l}$) without detectable rearrangement (A. E. Carnes and J. A. Williams, unpublished work).

Metabolic burden and plasmid production

The molecular mechanisms for the striking increased yield of plasmid DNA in this inducible fed-batch process are unknown. We speculate that one or more potential mechanisms contributing to yield improvement are: (i) reduced metabolic burden associated with plasmid replication during growth, (ii) reduced plasmid-mediated protein production during plasmid induction and (iii) altered DNA compaction during plasmid induction.

It has already been accepted that production of recombinant protein from plasmid places a metabolic burden on the host, diverting resources away from host metabolism [34]. The fermentation results described in the present study

strongly suggest that plasmid-mediated metabolic burden was responsible for the biomass inhibition and consequent low overall plasmid yields initially observed with the pUC-type plasmids. The present results observed at the cellular level indicated that reduced plasmid metabolic burden during the growth phase improved biomass production, which in turn provided more fodder for plasmid accumulation after temperature induction. Ideally, the metabolic apparatus of the cell would be shifted entirely towards plasmid production at this late stage, further reducing the volume of cellular debris through autolysis and energy recycling, salvage pathway activation and synthesis of plasmid DNA *de novo*.

Plasmid-mediated protein overexpression, and high copy plasmid production, both place a metabolic load on the host cell that limits growth and plasmid production [35,36]. In one plasmid fermentation process, the *kan^r* gene on a therapeutic plasmid produces 18% of the total cell protein, which imposes a significant metabolic burden [30]. SDS/PAGE protein gel analysis of total protein preparations from fermentation cells from the inducible process described herein detected high levels of the putative *kan^r* gene product. Interestingly, these levels were high in all samples and only modestly induced during growth at 42 °C, while the specific plasmid yield increased dramatically. Thus plasmid-mediated protein expression may be reduced after plasmid induction, reducing metabolic burden and increasing plasmid-carrying capacity.

Another possibility is that the process improves yield through alterations in the combinations of DNA compaction agents (e.g. histone-like protein or other chromatin-binding proteins, such as the *dps* (DNA-binding protein from starved cells) gene product [37]) present in the cells. Altered DNA condensation during the induction phase may increase plasmid yield by increasing tolerable plasmid levels or by increasing plasmid replication. Enhanced DNA compaction would also protect the DNA from endonuclease digestion [38], consistent with the observed low level of nicked plasmid obtained herein. Direct evidence of this (e.g. altered gel migration) has not been shown, however, and doing so may prove difficult, since the *in vivo* structure of DNA is subject to change upon lysis and isolation [39].

Theoretical plasmid yields

Studies with runaway plasmid R1 in a shake culture have achieved 75–80% total DNA as plasmid (up to three times relative to genomic). This may represent an upper limit for cell tolerance, as this is the maximum obtained with a variety of different sized plasmids, and is associated with viability loss and altered cell morphology [40,41]. Due to toxic elements in many plasmids, this maximum may not be obtainable with all plasmids. Also, *recA* production strains, used for propagation of therapeutic plasmids, may have a lower capacity, due to toxicity associated with the induction of

the SOS response (repair systems induced by the presence of single-stranded DNA) at high plasmid levels. However, the metabolic state of the cell, and other factors such as degree of DNA compaction and degree of plasmid-encoded gene transcription and/or translation will differentially affect the attainable theoretical limits.

Strain engineering to further improve plasmid production

Genomic, proteomic and/or metabolomic profiling technologies can be used to probe the underlying molecular mechanisms for yield improvement. RNA and/or protein preparations from controlled fermentation studies can be used to probe many of the 4500 or so genes' activities relative to one another. Metabolic flux analysis can identify altered or limiting metabolic products in the production hosts. Such analyses would allow the elucidation of RNA, protein or metabolic changes taking place in the inducible process, compared with conventional processes, and potentially the mechanism for improved yield.

In addition to providing clues to the basis for improved yield with the inducible fed-batch process, genomic and proteomic expression profiling studies may identify numerous potential targets for design engineering of superior strains for plasmid production. For example, genes involved in metabolism, biosynthesis and stress response to protein overproduction in *E. coli* have been identified by expression profiling [42]. A proteomic study identified a number of *E. coli* proteins whose expression is altered with increasing expression plasmid copy number (0, 36, 56 and 240 copy number evaluated), such as decreasing levels of translational capacity genes, including ribosomes, and increasing levels of heat-shock proteins [43]. Differential gene regulation under altered genetic or physiological conditions in fermentation culture has been observed [30,44–46].

This information gleaned from such studies may be used to rationally design engineered strains with improved performance. For example, Choi et al. [47] used DNA microarrays to identify a number of down-regulated genes during high-cell-density fed-batch *E. coli* fermentation producing insulin-like growth factor I fusion protein. This resulted in an engineered strain that increased protein yields from 1.8 to 4.3 g/l. Han et al. [44] used proteomic profiling to identify targets to strain engineer improved recombinant protein production. Engineered production strains with improved tolerance to the metabolic burden associated with high-copy-number plasmids may increase the plasmid-carrying capacity and overall fermentation yield.

Conclusions

The inducible plasmid fed-batch fermentation process reported herein results in improved plasmid productivity and

volumetric and specific plasmid yields 5–10-fold higher than previously reported [15,21,23,24]. Of further significance, the high specific plasmid yields obtained lead directly to higher final product purities. The combination of high yield fermentation and an exemplary purification process may provide cost-effective methodologies that further reduce genomic DNA to acceptable levels for gene therapy and DNA vaccination applications. Most importantly, however, improved yields and productivity will result in greatly reduced manufacturing costs.

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