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Inducible *E. coli* fermentation for increased plasmid DNA production

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Running title: Inducible plasmid fermentation

SUMMARY:

Bacterial plasmids are the vectors of choice for DNA vaccines and short term gene therapeutics. Growing plasmid DNA by microbial fermentation (*E. coli*) is usually combined with alkaline lysis/chromatography methods of purification. To date, optimal plasmid fermentation media and processes result in yields of 100-250 mg plasmid DNA/liter 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 productivity exceeding 1,000 mg plasmid DNA/liter was obtained after reduction of plasmid-mediated metabolic burden during growth. 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 five to ten fold increase in plasmid yield dramatically decreases plasmid manufacturing costs, and improves the effectiveness of downstream purification by reducing the fraction of impurities.

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

INTRODUCTION:

E. coli plasmids have long been the single most important source of recombinant DNA molecules used by researchers and by industry. Today, 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 may find application as preventive vaccines for viral, bacterial, or parasitic diseases; immunizing agents for the preparation of hyper immune globulin products; therapeutic vaccines for infectious diseases; or as cancer vaccines. 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 toward FDA approval, 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 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 [LPS] associated with gram negative bacteria), bacterial proteins, and RNA. Open-circle (nicked) and linear plasmid result from enzymic or shear-induced damage to supercoiled plasmid; these damaged plasmid isoforms must also be treated as impurities since they do not transfect eukaryotic cells efficiently [1]. Today, regulatory standards are not defined except in preliminary form [2]. 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, and such standards exceed the current purity attainable from established methods. Most glaringly, the FDA guidance “Points to Consider in the characterization of cell lines used to produce biologics” published in 1993 [3] recommends the standard of <100 pg host genomic DNA per dose (100 pg per 1mg 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% [4]). 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 (*e.g.* pBR322 with *rop* deletion) with or without a second site mutation that increases copy number (*e.g.* pUC, pMM1). Higher temperature (42°C) can be employed to induce selective plasmid amplification with pUC, and pMM1 replication origins [5, 6].

The use of reduced growth rate coupled with these high copy replication origins is the unifying principle in high quality, high yield plasmid fermentations [7, 8]. Generally, lower growth rates favor increased plasmid copy numbers [9, 10]. In a study to determine the effects of fermentation strategy on plasmid quality, O’Kennedy et al [11] found that higher growth rates in batch and fed-batch fermentations were associated with lower percentages of supercoiled plasmid. Lahijani et al. [12] have reported using a pUC origin plasmid in a fermentation with exponential feeding and a temperature shift from 37°C to 42-45°C. They achieved a plasmid yield of 218 mg/L. Schmidt et al [13] describe a fed-batch process using a glycerol yeast extract medium with DO-stat feedback controlled feeding producing up to 230 mg/L of plasmid. Chen [14] used a fed-batch process in semi-defined medium with combination DO-stat and pH-stat feedback control. This strategy led to a specific growth rate of 0.13 hr⁻¹ and plasmid yields of 82-98 mg/L. Durland and Eastman [15] 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 plateau at about 100-250 mg plasmid/L. This low yield imposes a cost and purity burden on commercialization of plasmid DNA production processes. We report herein the development of a novel inducible fed-batch fermentation process that breaks this plateau, producing 5-10 fold higher plasmid DNA productivity while maintaining plasmid integrity.

MATERIALS AND METHODS

Strains and Plasmids

Fermentations were typically performed with *E. coli* strain DH5 α [F- Φ 80*dlacZ* Δ M15 Δ (*lacZYA -argF*) U169 *recA1 endA1 hsdR17*(rk-, mk+) *phoA supE44* λ - *thi-1 gyrA96 relA1*], a widely used host for plasmid production. This strain 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 medium copy number and range in size from 5-8 kb. Plasmid pW2.0 is a 2.7 kb ampicillin resistance (*amp^r*) pUC19 derivative from our laboratory. Plasmids pmaxGFP (3.5 kb, Amaxa, Cologne, Germany) and pNTC-003040 (a 4.7 kb derivative from our laboratory) are kanamycin resistance (*kan^r*) plasmids with pUC replication origins. The plasmid gWiz GFP (Gene Therapy Systems, San Diego, California) is a 5757 bp *kan^r* pUC origin DNA vaccine plasmid vector.

Fermentation media

Proprietary semi-defined batch media (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 the nitrogen source. The trace metals and MgSO₄ concentrations have been optimized based on determined requirements of *E. coli* production strains. All components used in the media are well characterized and certified animal product free.

Fermentation Conditions

The seed cultures were started from glycerol stocks or colonies inoculated into LB medium plus 50 μ g/mL kanamycin or 100 μ g/mL ampicillin and grown in shake flasks at

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37°C. At mid-exponential phase (0.3-1.0 OD₆₀₀) the seed cultures were used to provide 1% inoculums 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 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 vessel volume per minute (VVM) and dissolved oxygen was maintained at 30% by proportional-integral control of agitation. At cell densities above about 20 OD₆₀₀, O₂ supplementation was also required and was increased automatically as needed during the rest of the fermentation to maintain 30% dissolved oxygen saturation.

During fed-batch fermentation a semi-defined feed nutrient was added according to a carbon limiting exponential feeding strategy. Briefly, an initial amount of carbon substrate is consumed during the batch phase at a specific growth rate of μ_{\max} . Upon exhaustion of the carbon substrate, the fed-batch phase begins and feed nutrient is added automatically at the rate determined by the following equation [16, 7]:

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

Where $F(t)$ is the feed rate, L/h,

μ = desired specific growth rate during fed-batch phase, hr⁻¹,

X_B = biomass concentration at the end of the batch phase, g dry cell weight/L,

V_B = initial liquid volume of culture, L,

S_f = limiting substrate concentration in nutrient feed medium, g/L,

$Y_{X/S}$ = yield coefficient of biomass from substrate, g/g,

t = time since beginning of fed-batch phase, hr.

Analytical Methods

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Culture samples were taken at key points and at regular intervals during all fermentations. Samples were analyzed immediately for biomass (OD_{600}) and for plasmid yield. Plasmid yield was determined by quantification of plasmid obtained from Qiagen Spin Miniprep Kit preparations. Briefly, cells were alkaline lysed, clarified, plasmid was column purified, and eluted prior to quantification. Cells were diluted in PBS and plated on LB+kanamycin media for live cell determinations. For cell morphology analysis, 10 μ L cell dilutions were incubated 5 minutes in the dark with 40 μ L PBS+10% glycerol containing 1/1000 dilution of SYBR Green I (Sigma, St. Louis, Missouri) and observed using fluorescence microscopy. Total DNA analysis was performed either using detergent lysis as described in Lin-Chao and Bremer [17] or phenol chloroform lysis as described in Williams et al [18]. Briefly, for phenol chloroform lysis, 0.5 OD_{600} units of cells were pelleted and resuspended in 200 μ L cell disruption buffer and disrupted by phenol chloroform extraction. Isolated plasmid and genomic DNA was resolved on 1% TBE agarose gels and detected by poststaining with a 1/10,000 dilution of SYBR Green I. The gels were photographed, scanned as JPEG documents, and genomic and plasmid DNA quantified using the Kodak 1D 3.6 program. The SDS and phenol chloroform lysis methods gave similar percent plasmid values. Total protein analysis by SDS-PAGE was performed as described in Williams et al [18]; 2.0 OD_{600} units of cells were pelleted and resuspended in 100 μ L of TE buffer, sonicated, mixed 1:1 with 2X sample buffer containing β -mercaptoethanol, and resolved on a 4-20% Tris HCl gel (Biorad, Hercules CA).

RESULTS:

pBR322-derived plasmid fermentation

Fermentation in NTC3018 (batch) and NTC3019 (fed-batch) media with a variety of medium copy number pBR322-derived plasmids were performed. Batch culture reached a cell density of 53 OD_{600} and plasmid yields of 57 mg/L while multiple fed-batch fermentations reached cell densities of 100-120 OD_{600} , (55-65 g dry cell weight per liter) with plasmid yield averaging 260 mg/L and reaching as high as 430 mg/L (**Fig. 1**).

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Importantly, the specific plasmid yields were very high, typically between 2.5 and 3.8 mg/L/OD₆₀₀, well exceeding levels observed with other fermentation media/processes using much higher copy pUC origin plasmids (**Table I**).

pUC plasmid batch fermentation

NTC3018 batch culture with pUC origin plasmids was then evaluated with pW2.0, pmaxGFP, and pNTC-003040. For pW2.0, the culture was grown at 37°C, and then amplification of plasmid copy number was induced by growth at 42°C late in the fermentation. The pmaxGFP culture was grown at 37°C for the entire cultivation time. The pNTC-003040 culture was grown at 34°C. Cell densities and plasmid yields are shown in **Table II**. While specific plasmid yields were slightly higher (2.9-5.3 mg/L/OD₆₀₀), overall productivity was lower (84-230 mg/L) due to reduced final biomass.

pUC plasmid fed-batch fermentation

The plasmid gWiz GFP was selected for fed-batch fermentation evaluation. Plasmid pmaxGFP was also tested and similar results were obtained.

Cell growth at 37°C in fed-batch fermentation stopped around 15 OD₆₀₀ (**Fig. 2a**). Plasmid yield analysis indicated an elevated specific plasmid yield of 2.7 mg/L/OD₆₀₀ 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 dropped to 1.6 mg/L/OD₆₀₀ and cell growth resumed; however, the specific plasmid yield gradually rose again and biomass growth stopped at about 60 OD₆₀₀ instead of growing to >100 OD₆₀₀ 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 (**Fig. 2b**), suggesting inhibition of cell division caused growth arrest, which would have likely eventually led to lysis [19]. After the temperature was reduced to 33°C a sample of growing cells showed much less filamentation (**Fig. 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 peaked at cell densities less than 60 OD₆₀₀. Biomass and plasmid DNA yield data 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 low, thus reducing the metabolic load. Temperature shifts to 37°C or 42°C were performed at an OD₆₀₀ of 60 to increase the plasmid copy number prior to harvest. Surprisingly, overall plasmid yields with gWiz GFP were 670 mg/L when shifted to 37°C, and 1100 mg/L when shifted to 42°C (**Fig. 3**). Specific plasmid yield and biomass increased for at least 9 hours following the temperature shifts. This strategy allowed the cultures to reach higher cell densities, ultimately exceeding 100 OD₆₀₀ with no filamentation or loss of cell viability.

Plasmid yields prior to the temperature shift remained low (< 2 mg/L/OD₆₀₀). Remarkably, the specific plasmid yields after temperature shift are very high, up to 12 mg/L/OD₆₀₀, well exceeding published yields (**Table I**). 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 (see above).

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; see below) to determine yields with an alternative plasmid, as well as to monitor plasmid DNA quality during the process. Induction was performed at an OD₆₀₀ of 53. Samples from throughout the fermentation were analyzed for plasmid DNA, total DNA, and total protein. The results are

summarized in **Table III**, and the total DNA profile from the samples is shown in **Fig. 3c**. The yield and purity were similar to those obtained with plasmid gWiz GFP. Plasmid DNA productivity was dramatically induced after the 42°C temperature shift, with a specific yield of 9.1 mg/L/OD₆₀₀ and overall yield of 751 mg/L 6 hours 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 hrs post 42°C induction). This represents an enrichment of plasmid content from 0.3x to 1.6x genomic DNA. Importantly, the fraction of monomeric plasmid was stable throughout the fermentation, and nicked or linearized versions of the plasmid were undetectable (**Fig. 3c**). Such low levels of nicked plasmid is unusual for plasmid fermentation processes, and may reflect enhanced DNA compaction and packaging that both increases cell carrying capacity and protects DNA from endonuclease digestion (see discussion). Collectively, these results demonstrate that the observed improved yield with the process is not plasmid specific and that the inducible process maintains plasmid stability and quality.

SDS-PAGE protein gel analysis of total protein preparations from the samples detected high levels of a 31 kDa protein in all samples; this protein was induced approximately 2 fold after 6 hours induction at 42°C. This protein correlates with the expected size and abundance of the plasmid borne kan^r gene product [20]. Additional proteins of approximately 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 (data not shown).

Process robustness

The inducible process was performed utilizing a series of seven Vaccine Research Center (VRC) pUC origin containing DNA vaccine plasmids each containing different target antigens (**Table IV**). The effect on plasmid yield of inducing plasmid production by shifting to 42°C at either 25-30 or 55-60 OD₆₀₀ was determined. The results (**Table IV**) demonstrate consistently high productivity when the induction is between 55 and 60

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OD₆₀₀ (5 out of 7 fermentations >500mg/L plasmid). Three out of five fermentations with induction at 25-30 OD₆₀₀ also exceeded 500 mg/L plasmid. The overall plasmid yield at harvest is lower when induced at 25-30 OD₆₀₀ (average yield is 525 mg/L versus average yield of 625 mg/L when induced at 55-60 OD₆₀₀) due to reduced overall biomass. The specific plasmid yield after induction was high when induced at either 25-30 or 55-60 OD₆₀₀.

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 0.5 gm/L in NTC3019 media, using the 30°C to 42°C inducible process in the DH5 α or DH1 cell lines (data 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 Vaccine Research Center (VRC) DNA vaccine plasmid (VRC 5737) in ten 10 L fermentation runs, all induced between 50 and 55 OD₆₀₀. The results (**Table V**) demonstrate high process consistency and productivity. No significant differences in specific plasmid yield were observed between 6 and 8 hours 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 are 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 (**Fig. 4**). This method, which achieves high specific plasmid yields *and* high cell densities, unexpectedly and dramatically improves (5-10 fold) the final overall yield of plasmid while maintaining plasmid integrity. The plasmid DNA produced with the process is high quality, being

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essentially 100% supercoiled with no detectable nicking, deletion or other rearrangement (**Fig. 3c**). The method is simple, is not limited to a specific host strain or plasmid backbone, nor does it require prescreening of individual colonies for high producing cell lines. While yields exceeding 1 gm per liter are reported with this method, we assume 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 a HPLC assay on total cell lysates.

The inducible fed-batch process described herein maintained low (<2 mg/L/OD₆₀₀) plasmid levels during the growth phase of the process before the temperature shift, and facilitated unprecedented ultra high plasmid production (6-12 mg/L/OD₆₀₀) after the temperature shift. Expressing plasmid yields in terms of specific yields (mg/L/OD₆₀₀) indicates 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 mg/L/OD₆₀₀ plasmid DNA accounted for 61% of total DNA or 1.6x chromosomal DNA (**Table III**); 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 (Carnes and Williams, Manuscript in Preparation).

O’Kennedy et al [21] 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 [22], widely used for initial large scale plasmid recovery. Although this fermentation process produces highly supercoiled plasmid, free of open-circle and linear isoforms, care must also be taken during downstream purification to prevent damage to the supercoiled plasmid. Plasmid is most susceptible to damage during the denaturation step of alkaline

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lysis; Clemson and Kelly [23] 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.

Palindrome sequences, direct or inverted repeats, and Z DNA forming sequences are deleted or rearranged by *E. coli* hosts. Some plasmids for therapeutic use must contain unstable sequences (*e.g.* inverted or direct repeats for shRNA therapeutics and for viral vectors such as AAV 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). 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. We have used this process to successfully produce long terminal repeat (LTR) containing plasmids, as well as deletion prone adenoviral associated virus (AAV) helper plasmids, in high yield (>0.5 g/L) without detectable rearrangement (Carnes and Williams, Manuscript in Preparation).

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 III**).

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:

- 1) reduced metabolic burden during growth
- 2) reduced plasmid mediated protein production during plasmid induction and
- 3) 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 [24].

The fermentation results described in this work 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 toward 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 productivity [25, 26]. 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 [20]. 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.

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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* gene product [27]) 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 [28], consistent with the observed low level of nicked plasmid obtained herein.

Theoretical plasmid yields

While we report herein plasmid fermentation yields of 61% supercoiled plasmid (1.6x plasmid relative to chromosomal DNA), studies with runaway R plasmids in shake culture have achieved 75-80% total DNA as plasmid (up to 3x relative to chromosomal). 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 [29, 30]. Due to toxic elements in many plasmids, this maximum may not be obtainable with all plasmids. As well, *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 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 4,500 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

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the elucidation of RNA, protein or metabolic changes taking place in the inducible process, compared to conventional processes, and potentially the mechanism for improved yield.

In addition to providing clues to the basis for improved productivity 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 [31]. 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 [32]. Differential gene regulation under altered genetic or physiological conditions in fermentation culture has been observed [20, 33-35].

This information gleaned from such studies may be used to rationally design engineered strains with improved performance. For example, Choi et al. [36] 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 g/L to 4.3 g/L. Han et al. [33] 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 productivity.

CONCLUSIONS

The inducible plasmid fed-batch fermentation process reported herein results in specific plasmid yields and overall plasmid yields five to ten fold higher than previously reported. Of further significance, the high specific plasmid yields obtained lead directly to higher final product purities. The combination of high yield fermentation and 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 will result in greatly reduced production costs.

ACKNOWLEDGMENTS

We would like to thank Sarah Langtry for media preparation, Steve Kelly for his tireless efforts cleaning, batching and operating the fermentors, and the NIH Vaccine Research Center for supplying the VRC DNA vaccine plasmids. Studies using the Vaccine Research Center's (VRC) proprietary plasmids were performed under a contract issued through and funded by the Intramural Research Program of the NIH, NIAID, VRC.

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Table I. Comparison of pUC origin plasmid fermentation yields

Fermentation process	Specific plasmid yield (mg/L/OD₆₀₀)*
Schmidt et al., 2003 (fed-batch)	1.3
Lahijani et al., 1996 (fed-batch)	2.8
NTC3018 pUC origin batch	2.9-5.3
NTC3019 pUC origin inducible fed-batch	6.5-12

*Expressing plasmid yields in terms of specific yields mg/L/OD₆₀₀ indicates the amount of plasmid relative to the total cell mass.

Table II. Yields from pUC plasmid batch fermentations with NTC3018 medium.

Plasmid	Final cell density (OD₆₀₀)	Overall plasmid yield (mg/L)	Specific plasmid yield (mg/L/OD₆₀₀)
pW2.0	57	230	4.0
pmaxGFP	16	84	5.3
pNTC-003040	57	163	2.9

Table III. Plasmid production kinetics

Sample	OD₆₀₀	Plasmid Yield (mg/L)	Plasmid Yield (mg/L/OD₆₀₀)	Plasmid Purity+ (% total DNA†)	Plasmid quality+ (% supercoiled monomer)
Flask (37°C)	0.8	-	-	22	-
Batch(30°C)	4	5	1.4	24 (26)	91
FB (30°C)	18	24	1.3	30 (33)	91
FB (30°C)	45	77	1.7	33 (36)	92
FB (42°C)	70	337	4.8	51 (55)	93
FB (42°C)	83	751	9.1	57 (61)	93

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

† Percent supercoiled monomer only. The supercoiled dimer plasmid comigrates with genomic. Numbers in brackets are estimated total supercoiled plasmid, based on percent supercoiled monomer.

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

Plasmid name	Target Antigen	Induction		Overall	
		OD₆₀₀	Final OD₆₀₀	yield (mg/L)	Specific yield (mg/L/OD600)
VRC4314	HIV gag	55	86	540	6.3
VRC4401	HIV gag	30	56	577	10.3
VRC4403	HIV pol	57	74	426	5.8
VRC4404	HIV nef	26	54	377	7
VRC5736	HIV Clade A envelope	26	66	334	5.1
VRC5737	HIV Clade B envelope	55	97	820	8.5
VRC5738	HIV Clade C envelope	27	62	606	9.8
VRC5738	HIV Clade C envelope	30	67	733	11

Table V. Data from ten 10L fed-batch fermentations with plasmid VRC 5737 showing process consistency.

Run #	Induction OD ₆₀₀	Final OD ₆₀₀	Overall				
			plasmid yield (mg/L)	Specific plasmid yield (mg/L/OD ₆₀₀)	Temp shift (hours)	Begin harvest (hours)	Time at 42°C (hours)
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

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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 1kb DNA ladder (Invitrogen, Carlsbad, CA) (left).

Figure 2. gWiz GFP plasmid fed-batch fermentation in *E. coli* with NTC3019 medium is shown: (a) growth and control parameter profile (dissolved oxygen, temperature, agitation) 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 reduced after the temperature was reduced to 33°C (right).

Figure 3. Inducible plasmid production process. gWiz GFP/ *E. coli* DH5 α inducible fed-batch fermentation in *E. coli* with NTC3019 medium (37°C or 42°C induction) is shown: (a) growth and plasmid productivity profile of a fermentation with a 30 \rightarrow 37°C temperature shift at 35 hours, plasmid yield reached 670 mg/L; (b) growth and plasmid productivity profile of a fermentation with a 30 \rightarrow 42°C temperature shift at 35 hours, plasmid yield reached 1100 mg/L; and (c) Total DNA analysis from sample timepoints of a VRC 5737 /*E. coli* DH5 α fermentation with a 30 \rightarrow 42°C temperature shift (see **Table III**). 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, OD₆₀₀ = 4 (lane 2), fed-batch phase fermentation, 30°C, OD₆₀₀ = 18 (lane 3), fed-batch phase fermentation, 30°C, OD₆₀₀ = 45 (lane 4), fed-batch phase fermentation, T=4 hrs post 42°C shift, OD₆₀₀ = 70 (lane 5), fed-batch phase fermentation T=6 hrs post 42°C OD₆₀₀ = 83 (lane 6). M is the 1kb DNA ladder (Invitrogen, Carlsbad, CA).

Figure 4. Inducible fed-batch fermentation process

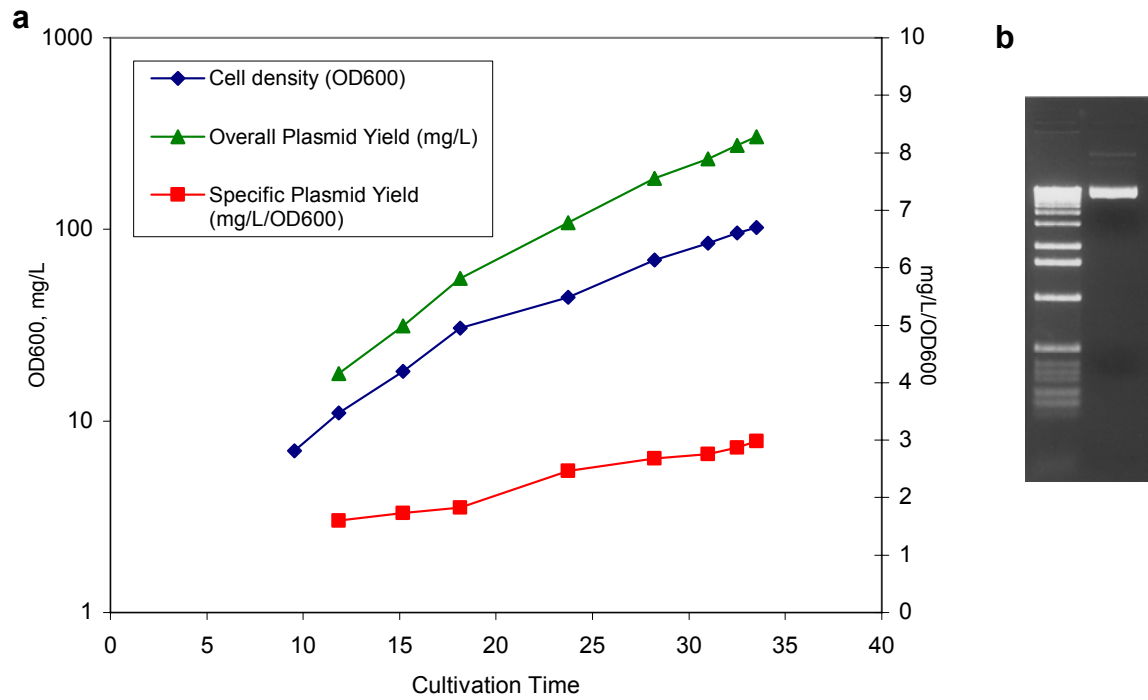


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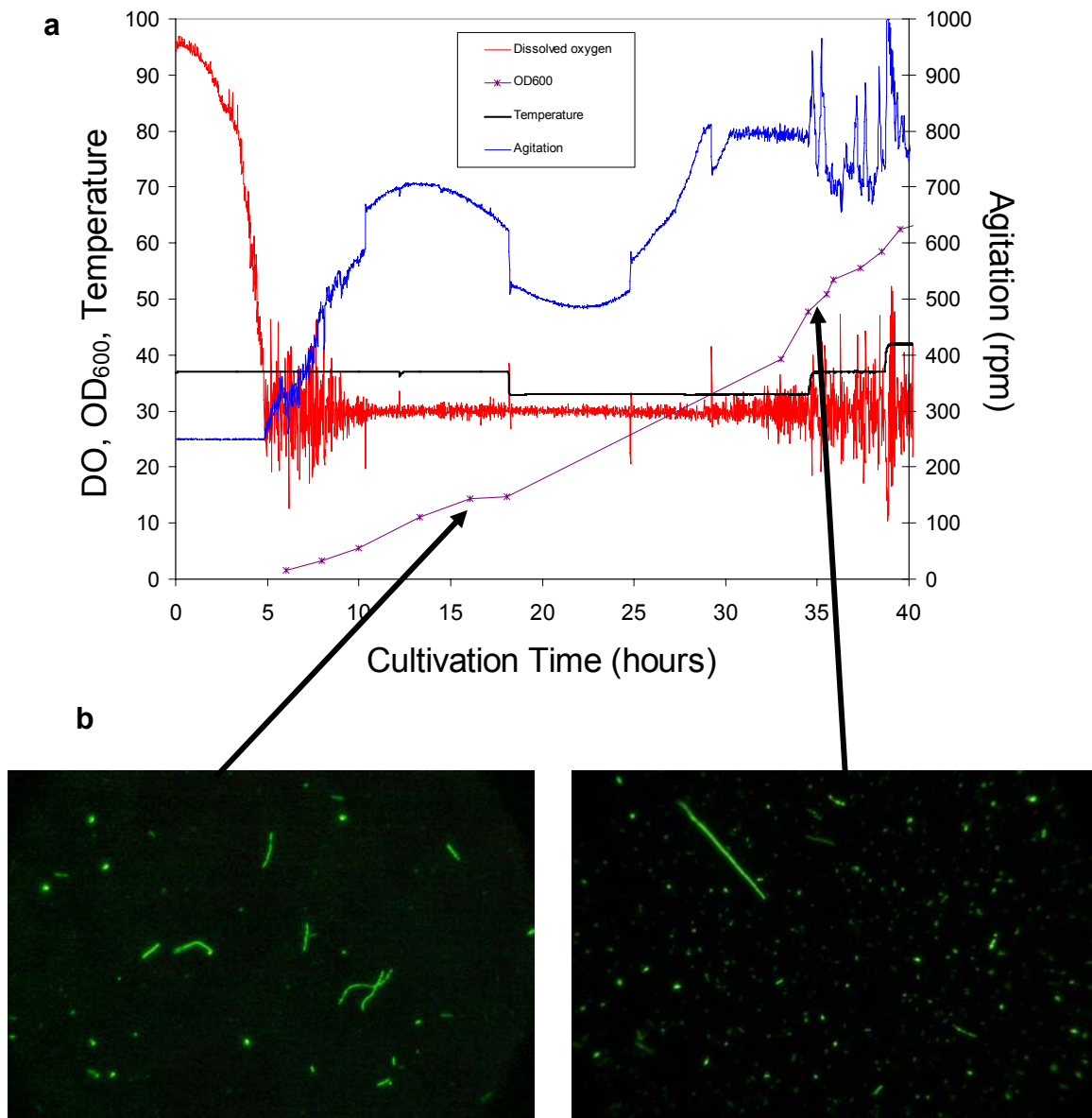


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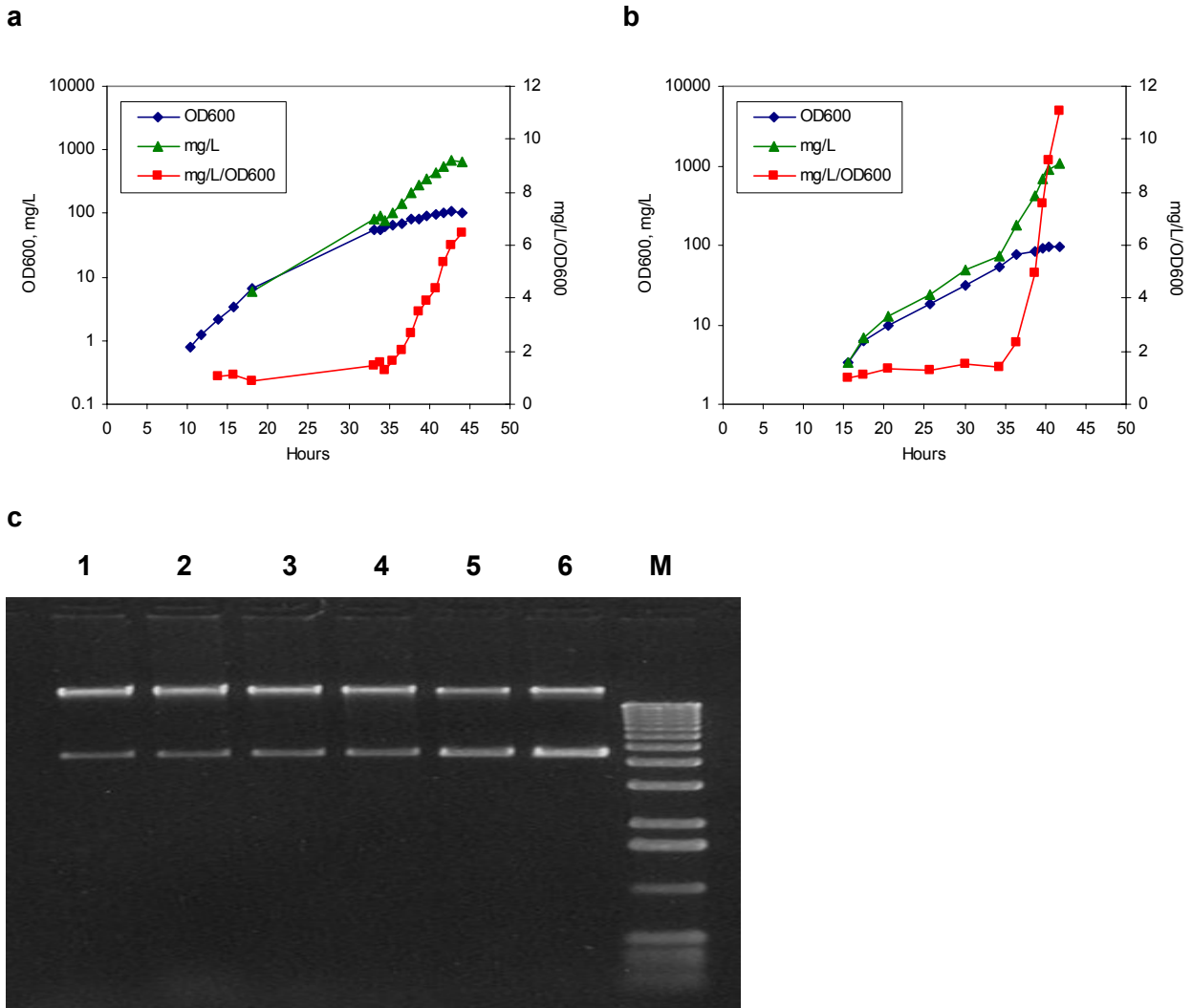


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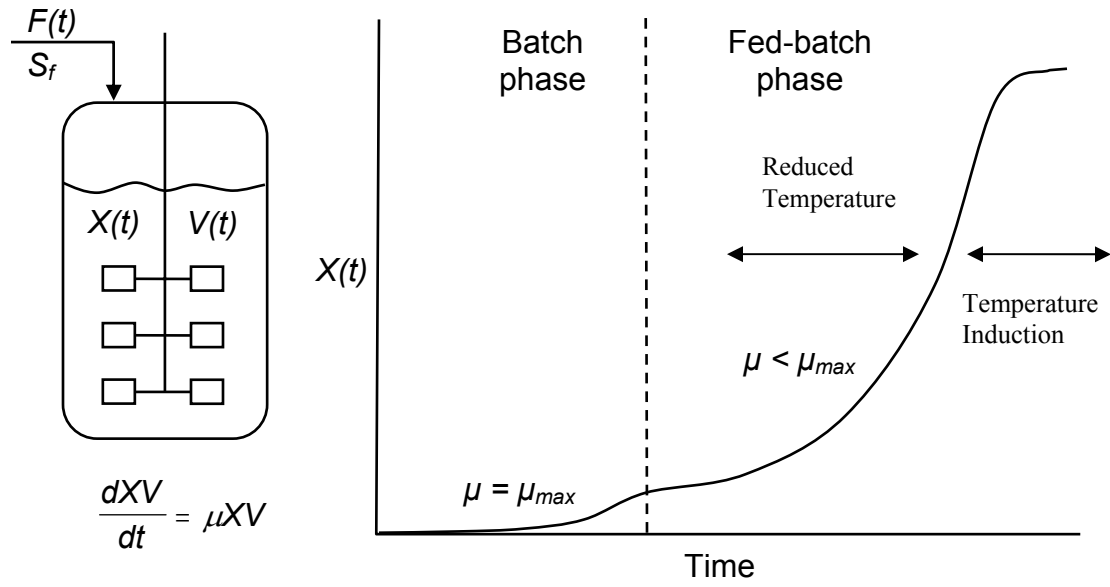


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