Escherichia coli Plasmid DNA Fermentation: Strain and Process-Specific Effects on Vector Yield, Quality and Transgene Expression

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Abstract

Industrial plasmid DNA manufacturing processes are needed to meet the quality, economy, and scale requirements projected for future commercial products. NTC has developed an inducible fed-batch fermentation process that incorporates novel cell bank and fermentation process innovations that reduce plasmid mediated metabolic burden. This process incorporates a scalable plasmid induction profile that, in combination with vector backbone modifications (PAS-BH-SV40 backbone; *e.g.* NTC8685 **Fig 3**) that double fermentation productivity compared to existing high copy vectors such as pVAX1 and gWIZ, form a generic plasmid DNA production platform with plasmid yields up to 2.6 g/L, and specific yields of 5% total dry cell weight.

The *dcm* methylase recognizes the internal cytosine residues in the recognition sequence 5'-CC*AGG-3' or 5'-CC*TGG-3' (**Fig. 3**, bars). This creates 5-methyl-cytosine (5mC), a common mammalian pattern (CG methylation) although the *dcm* methylated cytosine is in a different sequence context in bacteria. While plasmid production yields and quality are similar between *dcm*+ and *dcm*- host strains, CMV promoter expression is reduced by *dcm* methylation (**Fig. 4**). Surprisingly, despite improved expression, *dcm*- plasmid DNA is less immunogenic (**Fig. 5**). Our results demonstrate that it is critical to lock the plasmid methylation pattern (*i.e.* production strain) early in product development and that *dcm*- strains may be superior for gene medicine applications wherein reduced immunogenicity is desirable.

Materials and Methods

Strains and plasmids

E. coli DH5α: F- Φ 80d*lac*ZΔM15 Δ(*lac*ZYA -*arg*F) U169 *rec*A1 *end*A1 *hsd*R17(r_K-, m_K+) *pho*A *sup*E44 λ- *thi*-1 *gyr*A96 *rel*A1; NTC48107: DH5α *dcm*
Plasmid gWiz GFP: (Gene Therapy Systems) 5.8 kb, pUC origin, kanR
Plasmid NTC7485 (Williams *et al.,* 2006, 2009) 6.2 kb, pUC origin, kanR.
Plasmid NTC8685-EGFP, 3.9 kb, pUC origin, antibiotic-free selection (Luke *et al.,* 2009)

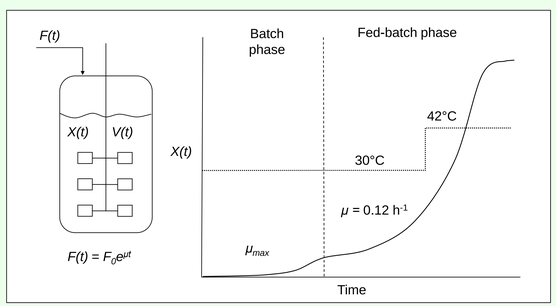


Fig. 1 Inducible fed-batch fermentation process (Carnes *et al.,* 2006)

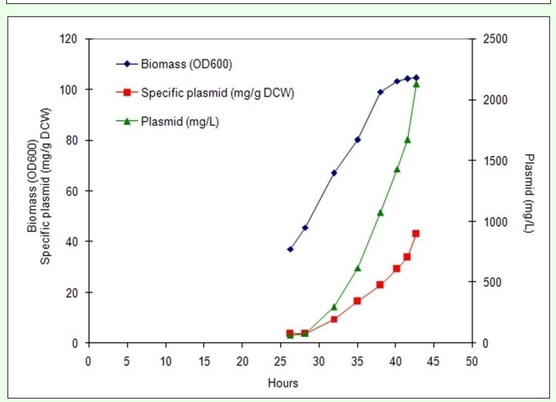


Fig. 2 NTC7485 plasmid production 30°C→42°C 2130 mg/L

Results

Manufacturing Process

This low metabolic burden inducible (30-42ºC) fed-batch fermentation process has successfully been scaled to 100L and 300L, and used for GMP production. Plasmid yields are superior to alternative processes (**Table 1;** Carnes, 2005; Carnes *et al.* 2006; Carnes and Williams, 2007; Williams *et al*., 2009). The temperature induction rate is not a critical process parameter; volumetric yields are not reduced using a slow ramp induction from 30ºC to 42ºC (**Table 1**). This insures scalability to industrial fermentors where temperature induction rates may be reduced compared to process scale bioreactors.

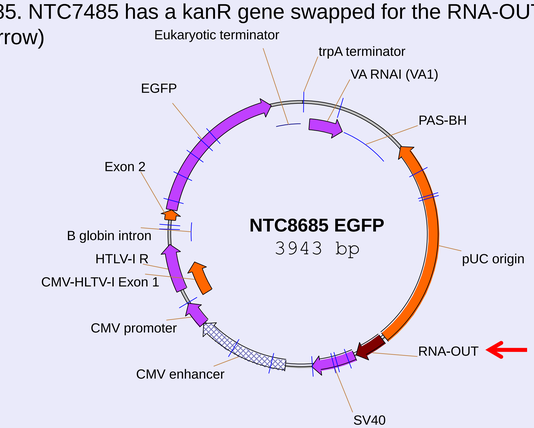
Reduced cell stress during the inducible fermentation process improves stability and yield of deletion prone long terminal repeat and short hairpin RNA (shRNA) vectors.

Table 1: Plasmid specific yields from fed-batch fermentation

Media *	Strain	Plasmid	Fermentation Process ‡	Density (OD_{600})	Volumetric yield(mg/L)	Specific yield (mg/L/OD_{600})
SD	DH5α	**NTC7485-derived**	30ºC growth 42ºC induction[1]	88	**2220**	**25** (51 mg/gDCW)
SD	DH5α		30ºC growth 42ºC **ramp** induction[1]	115	**2590**	**23**
SD	DH5α	gWiz-derived (kanR)	30ºC growth 42ºC induction[1]	97	1070	11
SD	DH5α	gWiz-derived (ampR)	30ºC growth 42ºC induction[2]	141	991	7
SD	BL21 (**dcm-**)	gWiz-derived (ampR)	30ºC growth 42ºC induction[2]	187	1923	10
D	DH5	pV1JNS derived	37ºC throughout[3]	90	1600	18 (39 mg/gDCW)

* SD = semi-defined; D = defined
‡ (1) Williams *et al.,* 2009, ramp induction is a slow temperature shift over 16 h
(2) Phue *et al.,* 2008, (3) Listner *et al.,* 2006.

Fig.3: Plasmid *dcm* methylation sites are shown (Blue bars) in antibiotic-free vector NTC8685. NTC7485 has a kanR gene swapped for the RNA-OUT sucrose selectable RNA (Arrow)



Dcm Methylation

Plasmids contain multiple *dcm* methylation sites (**Fig. 3**). The effect of *dcm* methylation on plasmid production and application was determined.

A *dcm*- derivative of DH5α (NTC48107) was created . Plasmid yield and quality was equivalent between *dcm*+ and *dcm*- strains in the low metabolic burden inducible (30-42ºC) fed-batch fermentation process. By contrast, eukaryotic cell expression was higher using *dcm*- plasmid DNA with both the CMV-HTLV-I R (NTC8685; **Fig 3**) and CMV (gWIZ, pVAX1) promoters (**Fig. 4**). Paradoxically, *dcm*- plasmid DNA for a NTC7485-derived vector encoding influenza hemagglutinin (HA) elicited lower anti-HA antibody responses in 6-8 week old BALB/C mice after intramuscular prime boost immunization with 10 µg naked DNA on day 0 and 21 (**Fig. 5**).

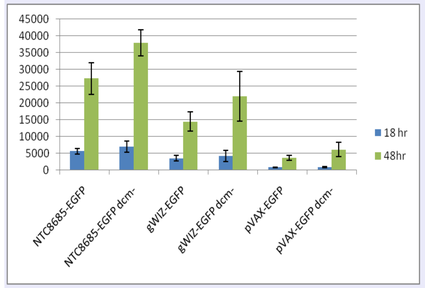


Fig.4: Plasmid *dcm* methylation reduces transgene expression in HEK293 (human)

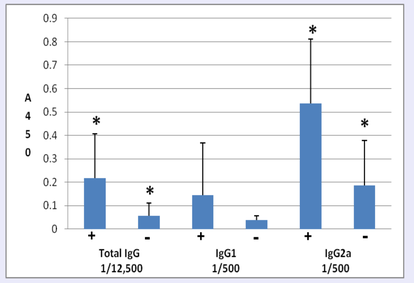


Fig.5: Plasmid *dcm* methylation increases transgene immunogenicity. Day 49 murine anti-HA humoral response after prime-boost

Conclusions

- The combination of optimized media, reduced temperature, and nutrient limited growth during biomass accumulation results in high process consistency and plasmid yields up to 2.6 g/L (5% DCW)
- Low metabolic burden seed bank and fermentation operation enable:
  - Production of previously toxic plasmids
  - Production of plasmids containing unstable sequences (*e.g.* inverted or direct repeats for shRNA therapies and for viral vectors such as AAV and HIV) eliminating need for stabilizing cell lines
- High specific plasmid yield increases final purity and downstream purification efficiency, dramatically decreasing manufacturing costs
- SV40- PAS-BH backbone 2 fold higher fermentation yield than gWiz
- Processes compatible with antibiotic-free vectors (Luke *et al.,* 2009)
- *dcm* methylation status affects expression and immunogenicity but not production
- *dcm*+ plasmid for DNA vaccination and *dcm*- for DNA therapeutics are recommended

Acknowledgements

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