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APPLICATION NOTE | No. 449

Optimizing Plasmid Yields – Impact of Flask Design, Media Type and Shaking Parameters

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Abstract

Recombinant plasmid DNA (pDNA) is produced in bacterial cultures, mostly in *Escherichia coli* (*E. coli*). Plasmid yield and quality depend on multiple factors, including the insert, the selection of host strain, the vector design and the methodology chosen for cultivation and downstream purification. In this application note, we have a closer look on how to optimize the cultivation of *E. coli* cultures in shake flasks. We examine the influence of culture media, vessel design, fill volume and shaking speed on bacteria and pDNA yields. We illustrate how larger production range can be obtained by using the appropriate combination between a large incubated shaker, optimized culture conditions and specifically-designed culture flasks.

Introduction

Plasmids serve as vehicles in genetic engineering either to clone and amplify DNA fragments, such as genes, or express recombinant proteins. Plasmid DNA (pDNA) can be easily genetically manipulated, produced in *E. coli* in large amounts and a variety of ready-to-use solutions allows easy subsequent downstream purification. Hence, they are a key component in all molecular biology applications, from academic research to biologic drug manufacturing up to the use as final drug product itself in new emerging gene therapy research and genetic vaccination [1,2].

Plasmids are small circular DNA molecules, naturally found in bacteria, that replicate independently from the host´s chromosomal DNA [3]. The gene of interest can be inserted into the plasmid by molecular cloning techniques. The resulting recombinant pDNA is introduced into the host cells, mostly *E. coli*, via transformation. The bacteria, that carry the plasmid, can be selected with help of the antibiotic resistance gene on the plasmid. For further expansion, shake flasks serve to either produce many identical copies

Innova® S44i Biological Shaker with Thomson Ultra Yield® Flasks

of the pDNA e.g. for functional analysis, for transfection into another host cell, or for inducing the expression of the gene for recombinant protein production.

Depending on the application, pDNA production ranks from research laboratory scale (up to a few milligrams) to industrial scale (milligram to gram scales). Plasmid yield depends on many factors. Differences between *E. coli* host strains can impact plasmid yields. *E. coli* JM109 and DH5α are popular strains for general cloning tasks and stable pDNA production, whereas BL21 is better suited for protein expression [4]. The plasmid vector, e.g. its' size and the number of copies they produce, has an impact, too. One differentiates low (15-20 copies/cell) and high copy (500-700 copies/cell) plasmids [5]. In general, cloning plasmids are high copy plasmids [6]. In protein expression applications a slowed down synthesis rate can have a positive impact on yields in case the protein is toxic, or protein aggregation is an issue, consequently, in this case, a low copy plasmid may be beneficial to use [7].

In this application note, we will examine the impact of cultivation with focus on high yield plasmid production. We will analyze the correlation between flask design, fill volume and agitation speed with different flask types and examine their influence on biomass and pDNA yields. We will also compare the impact of the culture media type.

Materials and Methods

Bacteria strains and plasmids: *E. coli* DH5α with pUC19 plasmid (Thermo Fisher Scientific) and *E. coli* JM109 with pGEM®-3Z plasmid (Promega). Bacteria were transformed according to manufacturer's instructions by heat-shock method. Positive recombinants were selected via blue/white screening from an agar plate in static culture. Glycerol stocks were prepared from liquid cultures and stored at -80°C. Media: Lennox-Broth (LB) medium (Invitrogen™, 12780052) and a nutrient rich media, modified Terrific Broth (TB). LB was prepared according to manufacturer's instructions. The modified TB was prepared with yeast extract (24g/L), tryptone (16g/L), casamino acids (10g/L), 1 % glycerol w/v and 100 mM MOPS buffer (pH 7.4). All media were freshly supplemented with 50 µg/mL ampicillin as selective antibiotic and an anti-foam agent 1:20.000 (Sigma) to reduce excess of foaming during shaking. Flasks: Erlenmeyer flasks, baffled and unbaffled (Corning®) and Ultra Yield® flasks (Thomson, Part # 931141 and 931136-B) with AirOtop™ Seals (Thomson, 899424 and 899425). All experiments were inoculated with the same start amount of 1 % (v/v) from a liquid seed culture and run in triplicates. Shaker: All experiments were performed in the Innova® S44i refrigerated incubated shaker with a 25 mm orbit at 37 °C. Read-out: Bacteria densities were measured by the OD 600 nm method with the Eppendorf BioSpectrometer® and UVette®. Bacterial biomasses were determined from harvested culture samples taken at different time points. For pDNA yield measurements, plasmid DNA was isolated with the PureYield™ Plasmid Miniprep System (Promega) according to manufacturer's recommendations. The pDNA concentration was measured by OD 260 nm using the Eppendorf BioSpectrometer and the µCuvette G 1.0. The pDNA was stored in Eppendorf DNA LoBind® Tubes.

Results and Discussion

Although *E. coli* is a facultative anaerobe, it grows best in the presence of oxygen [9]. Measures, that increase the oxygen supply, are beneficial for cultivation. There are different parameters one can adjust in cultivation to increase the aeration. On the shaker, e.g. the set agitation speed. On the vessel, the selected design and the fill volume.

Impact of flask design

Shake flasks are the most-commonly laboratory-scale cultivation vessels for production of plasmids. Different shake flask designs are available nowadays to increase the oxygen transfer to the culture [Figure 1]. The choice of the adequate flask design is depending on the organism's oxygen requirements and the individual application needs. In a first experiment, different flask designs were examined in small scale at different agitation speeds to select the best suited flask design for high yield production in larger volume flasks. Cultivation was done in rich media (modified TB).

Figure 1:
Left: Classic non-baffled and baffled Erlenmeyer flask design. Baffled flasks have defined cavities in the bottom area.
Right: Ultra Yield® flasks with steep vertical walls and six baffles at the flask bottom in 2.5 L size (Thomson).

Figure 2 shows, that the non-baffled flasks resulted in the slowest growth and lowest maximum cell densities compared to the baffled designs. An increase in agitation speed to 350 rpm did only slightly improve this. The classic baffled design improves the cell growth and maximum cell density, supported further by an increase in agitation speed. Best performed the Ultra Yield flasks reaching a maximum OD of 30 with both shaking speeds, with 200 rpm after 24 hours and with 350 rpm after 8 hours. Figure 2 shows that an increase in agitation speed is beneficial for all flask designs. Biomass measurements showed similar results (data not shown). The slightly better performance of the Thomson's Ultra Yield flasks compared to the standard baffled flask can be explained by the specialized design [Figure 1]. The manufacturer claims, that the specialized design increases aeration 10 x over standard shake flasks [8].

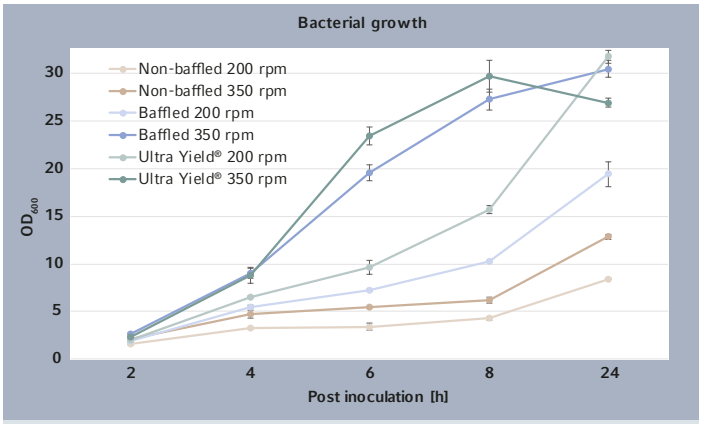

Figure 2: Influence of flask design and agitation speed on bacterial growth. Cultivation in rich media at 37 °C, flask size 500 mL with 25 % working volume.

The best performing design, the Thomson Ultra Yield flask, was used in following large scale-experiments to examine the impact of media type, fill volume and agitation speed.

Impact of media composition

The media supplies the culture with nutrients, such as proteins, minerals, vitamins and carbohydrates. Figure 3 and 4 show clearly the influence of the media composition on biomass and pDNA yields. The overall bacterial biomass in LB media stayed below 20 g/L in all cultivation settings, whereas the cultivation in the enriched TB media resulted in a 2–4-fold higher biomass up to > 60 g/L depending on working volume and shaking speed applied [Figure 3]. The resulting pDNA yields gave a similar picture. The pDNA yields from *E. coli* cultivated in LB media stayed below 10 mg/L in all conditions, whereas the incubation in rich media resulted in 4-5-fold higher yields [Figure 4].

Classic LB medium formulations are excellent for routine molecular biology applications, but bacteria growth is limited due to the only small amount of utilizible carbon sources containing. [10]. To achieve high yields with ODs ≥ 20, a buffered nutrient rich media is better suited. TB or modified versions contain beside a higher amount of yeast extract also glycerol as additional carbohydrate source and a buffer to stabilize the pH.

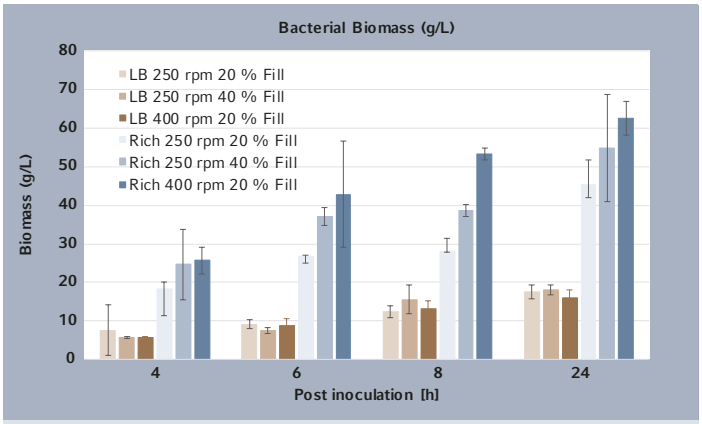

Figure 3: Bacterial biomass (DH5α with pUC19 plasmid) in 2.5 L Ultra Yield® flasks with different media, working volumes and agitation speeds incubated at 37 °C

Impact of agitation speed

Typical fill volumes for bacteria cultivations are 20–25 % with shaking around 200–250 rpm. The positive impact of a higher shaking speed seen already in small scale could be reproduced also in large scale rich media culture. The best results in biomass and pDNA yields gave the cultures with 20 % fill incubated at 400 rpm (the 40 % fill with 400 rpm resulted in cap wetting and was excluded from testing). Compared to the standard agitation at 250 rpm, an increase to 400 rpm resulted in a nearly 2-fold higher biomass after 8 hours [Figure 3] and a ~30 % increase in pDNA yields [Figure 4]. An increase in agitation speed supports the oxygen transfer to the culture supporting bacterial growth. In our experiments the higher biomass correlated also with higher pDNA yields.

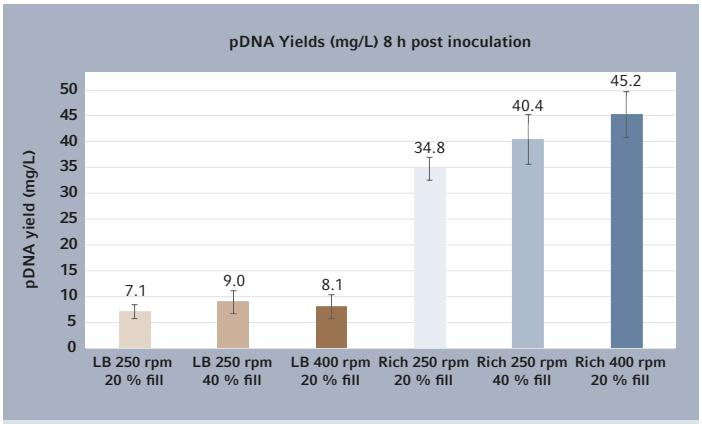

Figure 4: pDNA yields (DH5α with pUC19 plasmid) in 2.5 L Ultra Yield® flasks with different media, working volumes and agitation speeds incubated at 37 °C.

The positive impact of a higher shaking speed on biomass and pDNA yields could be reproduced with the second bacteria strain and plasmid construct in small-scale. An increase in agitation speed resulted in a 1.7-fold higher biomass [Figure 5] and a nearly 90 % increase in pDNA yields [Figure 6]. This experiment shows as well the impact of the strain and plasmid construct. The pGEM-3Z plasmid resulted in 2.6 fold higher pDNA yields compared to the pUC19 plasmid with similar biomass yields.

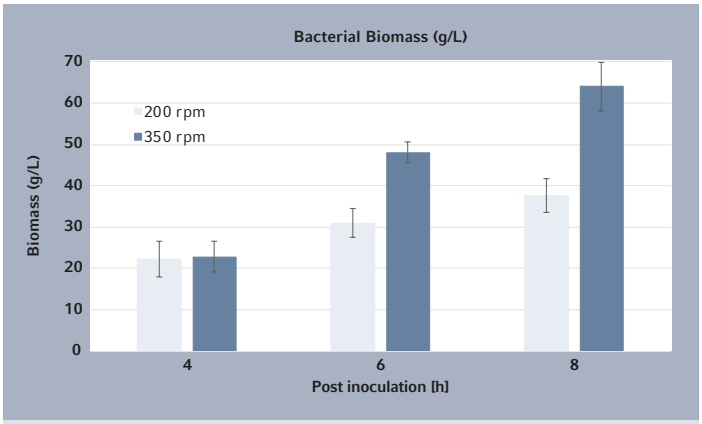

Figure 5: Bacterial biomass (*E. coli* JM109 with pGEM®-3Z plasmid) in 500 mL Ultra Yield® flasks with a fill volume of 25 % and different agitation speeds incubated at 37 °C in rich media after 4, 6 and 8 hours

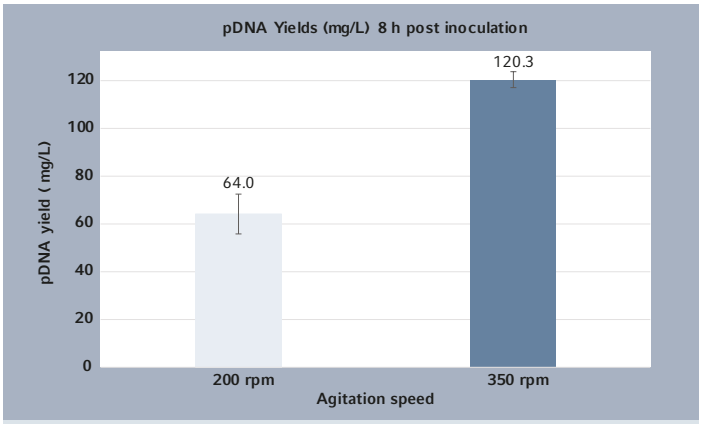

Figure 6: pDNA yields (*E. coli* JM109 with pGEM®-3Z plasmid) in 500 mL Ultra Yield® flasks with a fill volume of 25 % and different agitation speeds incubated at 37 °C in rich media after 8 hours

Impact of working volume

A larger culture volume can also improve plasmid yield. The higher fill volume (at same shaking speed of 250 rpm) resulted in higher yields in the rich media culture. Doubling the culture volume from 20 to 40 % resulted in a ~1.4 higher biomass [Figure 3] and an increase of pDNA yields > 15 % after 8 hours [Figure 4]. This is a bit unexpected, as usually a higher fill results in oxygen limitation. One explanation may be the specific baffled design and flow behavior in the specialized Ultra Yield® designs.

Impact of the shaker design

Following selection criteria are useful when selecting a shaker for high yield plasmid production:

Shaker capacity: The shaker should have a high vessel and weight load capacity. Stackable incubated shakers further increase capacity per footprint. Depending on fill volume, a volume between 19.5 (20 % fill) – 45 L (40 % fill) can be produced using 2.5 Liter Ultra Yield® flasks in a triple stacked large incubated shaker, here taken as example the capacity of the Eppendorf S44i [Table 1].

Platform type	Flasks/platform	Volume/flask	Volume/shaker	Volume/triple-stack shaker
Universal	13	0.5–1 L	6.5–13 L	19.5–39 L
Dedicated	15	0.5–1 L	7.5–15 L	22.5–45 L

Table 1: 2.5 L Ultra Yield flask apacity and typical cultivation volumes on universal and dedicated platform of the Eppendorf S44i shaker.

Theoretical calculated yields using 2.5 Ultra Yield flasks may result in ~783 to 1800 mg of pDNA in a triple-stacked configuration, taken as example the numbers from the experiment with the pUC19 plasmid [Table 2].

	Results from experiment (mg/L) [Figure 4]	Resulting pDNA (mg/flask)	Resulting pDNA (mg/shaker)	Resulting pDNA (mg/triple-stack)
20 % Fill, 250 rpm	34.8	17.4	261.0	783.0
20 % Fill, 400 rpm	45.2	22.6	339.0	1017.0
40 % Fill, 250 rpm	40.4	40.4	606.0	1818.0

Table 2: Theoretical calculated pDNA yields with the different cultivation conditions per shake flask, per single and per triple stacked shaker in the Eppendorf S44i equipped with fully loaded dedicated platform (15 flasks/platform). Values based on the experiment with *E. coli* DH5α with pUC19 plasmid in rich media in 2.5 L UY flasks [Figure 4].

Shaker drive robustness
The shaker should operate reliably with high weight loads also at high shaking speeds. Shakers with a multi-shaft drive system, like the triple eccentric drive or the Eppendorf X-Drive, stabilize the platform on more than one point, providing maximum stability also at high speed-load scenarios. To compensate the centrifugal force created by the liquid mass, the shaker drive should be equipped with a good counterbalance. A poorly counterbalanced can not only lead to an imbalance situation, but also to a higher wear out of the shaker over time. An adjustable counterbalance is the most flexible, semi-automatic systems are the most convenient and allow precise adjustment.

Optimal temperature control and programming
Besides shaking performance, optimal temperature control is a factor to look at. An incubated shaker gives usually more precise temperature control than an open air shaker operated in a climate room. It may be equipped with refrigeration if the application requires an incubation below room temperature. And multiple-step programming permits easy scheduling of defined growth windows by inserting e.g. a cooling step before or after the optimal growth temperature. This may also enhance reproducibility of the culture.

Conclusion

Optimizing the bacteria cultivation conditions can be one set-screw in enhancing pDNA yields. Using a nutrient rich media instead of LB, baffled or specialized flask designs like Ultra Yield flasks and an increase in shaking speed can positively impact bacterial growth and thus subsequent product yields. Hence, selection criteria for shakers should be beside capacity, a robust drive and counterbalance system to operate reliably with high weight and shaking speeds. Preprogramming of temperature and rpm allow convenient and reproducible cultivation in defined growth windows.

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Innova® S44i, refrigerated, orbit 5.1 cm (2 in), Japan, 100 V, 50/60 Hz	S44I330006
30.7 cm (12.1 in) positioning base	S44I041002
Universal platform	S44I040001
500 mL Erlenmeyer clamp	M1190-9003
2000 mL Erlenmeyer Clamp, for narrow neck Erlenmeyer (DIN-ISO 1773) or Thomson flasks	M1190-9005
Eppendorf BioSpectrometer® kinetic	6136 000 800
Eppendorf UVette® 220 nm – 1,600 nm	0030 106 300
Eppendorf µCuvette G 1.0	6138 000 018
Eppendorf DNA LoBind® Tubes	0030 108 051

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