APPLICATION NOTE No. 448

E. coli Plasmid DNA Production Using the DASbox® Mini Bioreactor System

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Abstract

Optimal plasmid DNA production requires a cost-efficient, high yield processing technology.

Due to the increasing demand for mRNA vaccines and gene therapy vectors, large-scale plasmid DNA (pDNA) production is now common. While plasmid DNA production is often carried out through large-scale industrial fermentations protocols, process development is usually conducted in small to bench scale fermenters. In high yield plasmid production processes, optimization of the plasmid vectors, favorable interaction between plasmid and the host organism, and improvement of the growth environment are crucial for the success.

In this study, we have cultivated *E. coli* DH5α using the Eppendorf DASbox Mini Bioreactor System, and we compared a batch and fed-batch processes.

The objectives of this study were (1) to demonstrate the feasibility of plasmid DNA production in a bioprocess using the DASbox Mini Bioreactor System, and (2) to compare fed-batch with batch culture with respect to plasmid DNA yield and process efficiency.

Introduction

In recent years, the need for improved high-yield plasmid DNA production strategies has significantly increased due to its application in the field of messenger RNA (mRNA) vaccines [1] and gene therapy [2]. In order to fulfill the demand for pDNA for use in the biopharmaceutic industry, upstream processing must be optimized to maximize the amount of pDNA produced, which could be accomplished by maximizing the final cell concentration and the average plasmid copy number [3]. As the plasmid titer is directly related to biomass formation, fermentation strategies are based upon increasing total cell density [4].

An fed-batch fermentation using the well characterized host cell *E. coli* DH5α results in an enhanced growth rate and the ability to achieve high cell densities with minimal nutrient requirements, with resultant lowered manufacturing costs [5].

Besides high purity DNA preparation, high concentration is also a prerequisite for optimal production values. The aim of a production process is to limit contaminants such as genomic DNA (gDNA) or RNA and to work towards a unified conformational structure of the plasmid DNA [6]. In previous studies, it has been found that the contamination with gDNA can be significantly reduced by adopting fed-batch culture over a batch culture [6].

In this study, we outline the feasibility of plasmid production in the Eppendorf DASbox Mini Bioreactor

System. Our goal was to increase the overall yield compared to previous studies done with Eppendorf Conical Tubes [7]. We also analyze the effect of a fed-batch process as compared to a batch process.

Material and Methods

E. coli strain and glycerol stocks

E. coli DH5α DSMZ (6897), purchased from the Leibniz institute DSMZ – German Collection of Microorganisms and Cell Cultures GmbH, is a wildtype strain which naturally hosts multiple copies of plasmids. Genetically modified variants of this strain are commonly used for pDNA production [5] .

We reactivated the freeze-dried cells in 50 mL Lysogenic Broth (LB medium, Table 1) in Erlenmeyer flasks without baffles overnight at 37 °C, 200 rpm in a New Brunswick Innova® 42 incubator. The next morning, we transferred 10 mL of the overnight culture to 100 mL of fresh, sterile LB-medium in a 1000 mL Erlenmeyer flask without baffles. This culture was further incubated at 37 °C with 200 rpm.

After 5 hours of growth, we checked the culture for possible contamination using a Zeiss® Primo star microscope.

To prepare glycerol stocks, we measured the OD_{600} of the culture, mixed 825 µL broth with 125 µL 70 % (w/v) Glycerol to a final OD_{600} of 3.5 per vial. The stocks were frozen and individually thawed at room temperature for use.

Fermentation system

In this study, we used the DASbox Mini Bioreactor System for microbial applications. More details are described in Figure 1. We used the bioprocess control software DASware® control 6.

The DASbox Mini Bioreactor System is a parallel bioreactor system, which allowed us running batch and fed-batch fermentations simultaneously.

Medium preparation

Two different growth media were used in this study, for preparation of the inoculum and for the fermentation phase.

Complex medium for inoculum preparation

We used Lysogenic Broth (LB medium, Table 1) for preparation of the pre-cultures and the inoculum. The

Fig. 1: DASbox Mini Bioreactor System

Setup of fermentation system

In this study, we used a DASbox Mini Bioreactor System for microbial applications. It was equipped with autoclavable 250 mL glass vessels with Rushton type impellers. In addition to the routinely used sensors for pH, DO, and temperature we installed level sensors to the vessels to allow for the automatic addition of antifoam.

To know more about the possibilities of configuring the DASbox system tailored to your needs, visit
www.eppendorf.group/dasbox

Table 1: Lysogenic broth (per liter)

Tryptone	10 g
Yeast extract	5 g
Sodium chloride	10 g
Deionized water	ad 1 L
	Sterilize by autoclaving

medium was prepared by dissolving 10 g tryptone, 5 g yeast extract and 10 g sodium chloride per 1 L deionized water, and autoclaving for 20 minutes at 121 °C.

Media for batch fermentation

To prepare the PAN-medium used for batch fermentation we prepared the following stock solutions to separate heat sensitive from autoclavable components.

Table 2: 10x PAN medium stock solution (per liter)

Calcium chloride dihydrate	0.15 g
Potassium dihydrogen phosphate	30 g
Dipotassium hydrogen phosphate	120 g
Ammonium sulfate	50 g
Iron(II) sulfate heptahydrate	0.75 g
Trisodium citrate dihydrate	10 g
Deionized water	ad 1 L
	Sterilize by autoclaving

Table 3: Magnesium sulfate stock solution (per liter)

Magnesium sulfate heptahydrate	100 g
Deionized water	ad 1 L
	Sterilize by autoclaving

Table 4: PAN trace elements solution (per liter)

Aluminium sulfate octadecahydrate	2.0 g
Cobalt(II) sullfate heptahydrate	0.8 g
Copper(II) sulfate pentahydrate	2.5 g
Boric acid	0.5 g
Manganese sulfate monohydrate	24 g
Sodium molybdate dihydrate	3.0 g
Nickel(II) sulfate hexahydrate	31.5 g
Zinc sulfate heptahydrate	15 g
Sulfuric acid, 25 %	2.4 g
Deionized water	ad 1 L
	Sterilize by autoclaving

Table 5: Thiamine stock solution (per liter)

Thiamine hydrochloride	5 g
Deionized water	ad 1 L
	Sterilize by 0.2 µm filtration

To prepare the final medium, we transferred 10x PAN-medium stock solution, 10 % Struktol J-673 and diH_2O individually into each bioreactor and heat-sterilized them for 20 min at 121 °C by using a Systec VE-95 autoclave in the wet cycle settings.

Table 6: 1x PAN medium with additions per vessel (to reach 150 mL initial volume)

10x PAN medium stock solution	15.0 mL
10 % (w/v) Struktol J-673	3.0 mL
Deionized water	111.7 mL
Add components to the vessel and sterilize by autoclaving. After cooling, add the folowing heat-sensitive components through a feed tube using a 0.2 µm sterile filter	
Magnesium sulfate stock solution	0.5 mL
50 % glucose solution	12.0 mL
Thiamine stock solution	0.2 mL
PAN trace elements solution	0.2 mL

After cooling to room temperature, we added the heat-sensitive components (see Table 6). The final initial volume of 150 mL per vessel was reached after inoculating with 7.5 mL preculture 2.

Additionally, a solution of 10 % (w/v) Struktol® J-673 was prepared to serve for foam dissolution as well as a 10 % ammonium hydroxide solution for pH correction.

Feed medium

The feed medium consists of 50 % Glucose solution with additions (Table 7).

Table 7: 50 % (w/v) glucose solution with additions (per liter)

Glucose monohydrate	550 g
Ad 1 L deionized water and sterilize by autoclaving. After cooling, add the following heat-sensitive components through a 0.2 µm sterile filter	
Thiamine hydrochloride	1 mL
PAN trace elements solution	1 mL

Solutions for plasmid DNA extraction

In order to quantify the yield of pDNA the standard method of alkaline lysis was done according to application note 427 by Eppendorf [7].

We prepared the solutions 1, 2, and 3 for pDNA extraction (Table 8, 9, 10). Additionally, an aliquot of isopropanol was placed in the freezer to have ready to use and cold for extraction.

Vessel setup

In this study, we performed feed addition submersed and the base and antifoam addition in the reactor's headspace. To be able to control the addition of antifoam, a level sensor was installed via an additional triple port.

Table 8: Solution 1 for plasmid extraction (per liter)

Glucose monohydrate	9.91 g
Ethylenediaminetetraacetic acid (EDTA)	2.92 g
Tris (pH 8.0)	3.02 g
Next component needs to be added immediately before usage	
RNase A	100 mg

Table 9: Solution 2 for plasmid extraction (per liter)

Sodium hydroxide	0.008 g
Sodium dodecyl sulfate (SDS)	0.288 g
Deionized water	ad 1 L

Table 10: Solution 3 for plasmid extraction (per liter)

Potassium acetate	294.42 g
Sodium dodecyl sulfate (SDS)	0.288 g
Deionized water	ad 1 L

Table 11: 10 x TE-buffer (per liter)

Tris-base	6.05 g
Adjust to pH 8.0 with concentrated hydrochloric acid	
Ethylenediaminetetraacetic acid (EDTA)	2.92 g

We installed pH and DO sensors in the Pg 13.5 threads of the head plate and used the sample port of the bioreactor for inoculation. The headplate assignment is illustrated in Figure 2.

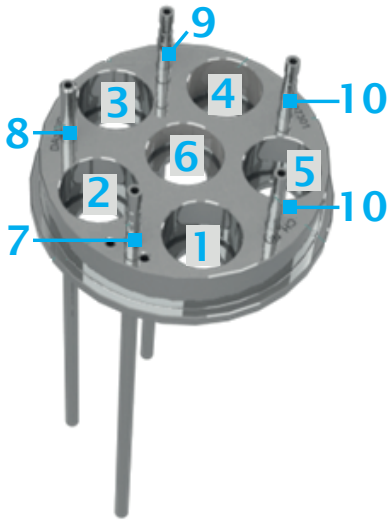
Inoculum preparation

Before inoculum preparation, we selected one cryovial of the previously prepared glycerol stocks to thaw at room temperature.

We transferred 50 mL sterile LB medium to each of two sterile 500 mL Erlenmeyer flasks without baffles. With 1 mL of the thawed glycerol stock we inoculated one of the Erlenmeyer flasks. The second Erlenmeyer flask served as a sterile control.

The culture was grown in an Innova 42 shaker overnight at 37 °C, 200 rpm. Subsequently we initiated a second pre-culture using 10 mL of the overnight culture and 100 mL fresh LB-Medium in a 1000 mL Erlenmeyer flask without baffles.

After incubation for 8 h at the same shaker settings, we



	Port	Port accessory	Associated device	Purpose
1	Pg 13.5	-	DO sensor	DO monitoring
2	Pg 13.5	-	pH sensor	pH monitoring
3	Pg 13.5	Triple port position 1 Triple port position 2 Triple port position 3	Level sensor Short dip tube Short dip tube	Foam monitoring Free Free
4	Pg 13.5	Compression fitting I.D. 12 mm	Exhaust condenser	Exhaust treatment
5	Pg 13.5	Compression fitting I.D. 4 mm	L-sparger	Gassing
6	Pg 13.5	-	Lipseal stirrer assembly	Agitation
7	Long dip tube	-	Sample port	Inoculation and offline sampling
8	-	Thermowell	Platinum RTD temperature sensor	Temperature monitoring
9	Long dip tube	-	Long liquid addition port	Feed addition
10	Short dip tube	-	Short liquid addition port	Base addition; antifoam addition

I.D.: inner diameter

Fig. 2: Headplate assignment of a DASbox glass vessel using in this study. Pg 13.5 port assignment is flexible (except for the agitation assembly)

measured the OD_{600} with an Eppendorf BioPhotometer® D30 to be within our target range of 6 – 8.
Afterwards we inoculated the vessels individually with 5 % (7.5 mL) of the initial reactor volume.

Sensor calibration

We calibrated the pH sensors in parallel outside of the vessel, before sterilization, following the 2-point calibration procedure of the DASware control 6. We used a pH buffer 7.0 to set the probe's offset and a pH buffer 4.01 to set the slope.

Before we calibrated the DO sensors and prior to autoclaving the vessel, we filled the sensor cap with fresh electrolyte solution.
We recalibrated the DO sensors in parallel inside the vessel after sterilization and addition of the medium supplements. A period of 6 hours is needed to polarize the sensors by connecting them to the control station. The control station provides a polarization voltage to establish an anode and a cathode within the sensor.

It is recommended to set the reactor conditions during DO calibration to the initial set points of the fermentation. Under the conditions adopted in this protocol, that equals a stir rate of 400 rpm, a gas flow of 0.5 sL/h and a temperature of 37 °C.

We did the 2-point calibration by sparging with pure nitrogen first to set the offset (0 % DO) followed by sparging in air to calibrate the slope (100 % DO).

Pump calibration

Before connecting the vessels to the fermentation system, we performed the pump calibration following the procedure of DASware control 6. We calibrated the feed pumps in parallel by using the feed medium and adjusting the density to 1.12. We calibrated the base-pumps in parallel using water as the calibration solution.

Feed start and feed profile

For the fed-batch culture, we set up the feed profile and its control via a reactor script in DASware control 6.

The feed start was triggered based on detection of a DO spike. At the end of a delay period of 1 h after inoculation, the DO value will reach 30 %. Once a threshold of 40 % DO is reached with rising edge, the pump used for feeding is started and the feed profile is executed.

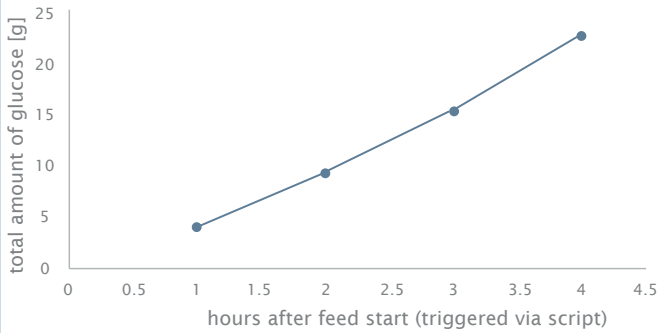


Fig. 3: Feed profile used during fed-batch phase.

We used a stepwise feeding schedule based on an exponential feed. The calculations were derived from the specific growth rate of the *E. coli* strain DH5α from previous experiments (see Table 12 and Figure 3).

In total, the fed-batch phase of fermentation took 5 h.

Process parameter setup

The batch and fed-batch setups used the parameters listed in Table 13.

We designed the DO cascade to maintain the dissolved oxygen at 30 % by first accelerating the agitation from 400 to 2000 rpm, then increasing the air flow rate from 4.5 sL/h to 18 sL/h (0.5 – 2 vvm for 150 mL initial volume), and finally enriching the oxygen concentration in the gas flow from 21 % to 100 %. These three steps correspond to the DO output of 0 % – 50 %, 50 % - 75 % and finally 75 % - 100 % (see Figure 4).

Optical density measurements

We measured optical density using an Eppendorf BioPhotometer D30 at a wavelength of 600 nm. We took samples regularly during the fermentation.
To achieve an appropriate measurement in the linear range

Table 12: Feed profile used for fed-batch fermentation

Hours after feed start	Pump rate [ml/h]	Glucose amount fed in this step [g]	Total amount glucose fed [g]
1	7.50	4.125	4.125
2	9.08	4.994	9.119
3	11.19	6.154	15.273
4	13.86	7.632	22.905
5	16.75	8.375	31.280

Table 13: Process parameters used for the fermentation

Parameter	Configuration
Vessel	DASbox Mini Bioreactor for microbial applications
Inoculation density	5 % of initial volume (OD_{600} of inoculum was 6-8)
DO	30 %, maintained by DO cascade
Agitation	Overhead drive, maximum 2000 rpm, controlled by DO cascade
Gassing	Automatic gas flow and mix, controlled by DO cascade
Temperature	37 °C, heating and cooling done by Peltier elements in the reactor's positions
pH	7.0, one-sided control with 10 % (v/v) sterile ammonium hydroxide solution
Antifoam	10 % sterile Struktol J-673, control via level sensor
Sparger	L-sparger
Feeding	Automatically triggered via reactor script

between 0.2 and 0.8, we diluted the samples accordingly.

Determination of cell wet weight (CWW)

To determine the CWW, we transferred 1.5 mL of sample into a 2 mL Eppendorf tube. The tare weight of the empty vial was determined previously using an analytical scale. We centrifuged the samples at 16,220 x g for 10 min using the Eppendorf Centrifuge 5427R. The vials were decanted after centrifugation and the weight of the Eppendorf tube with pellet was measured to calculate the final net weight of biomass.

The vials containing the pellet were stored at 4 °C until the end of fermentation to proceed with the pDNA extraction in batch.

Plasmid DNA extraction

We performed the plasmid DNA extraction following the protocol for alkaline lysis described in Application note 427 by Eppendorf [7].

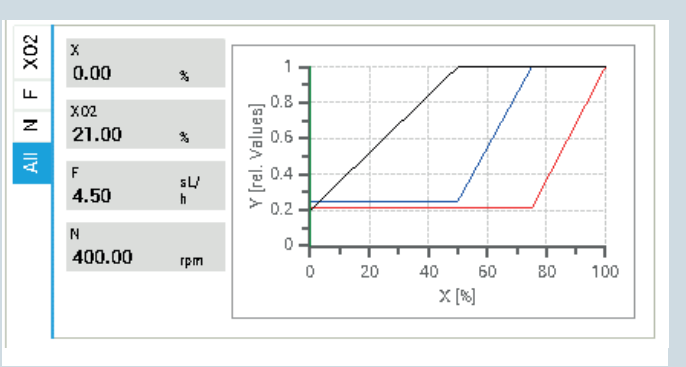


Fig. 4: Visualization of the DO cascade built in DASware control 6. First, the stirrer speed is increased (black curve) followed by the gas flow (blue curve) and finally oxygen is enriched (red curve)

The stored *E. coli* pellets were set to reach room temperature the next day. We resuspended the pellets in 0.3 mL solution 1 (Table 8). After 5 min incubation, 0.6 mL of solution 2 (Table 9) was added and the samples were mixed by inverting and further incubated for 10 min on ice. After addition of 0.45 mL of solution 3 (Table 10) we mixed the samples and centrifuged them at 16,220 x g for 30 min at 4 °C.

We transferred the supernatants to new tubes, precipitated with same volume of cold isopropanol, mixed and centrifuged at 16,220 x g for 30 min at 4 °C.

Afterwards we rinsed the pellets once with 70 % Ethanol, centrifuged, dried and resuspended them in 40 µL TE buffer (Table 11) for measurement in the BioPhotometer D30 from Eppendorf.

We estimated the yield of pDNA by measuring the absorbance of the prepared sample at 260 nm. Additional measurements at a wavelength of 230 nm and 280 nm were done to assess the purity of the established DNA preparations.

Results

We ran the fermentation of *E. coli* DH5α in parallel in a batch setup and in a fed-batch set up. We maintained the DO by applying a customized DO cascade (Figure 4). The feeding for fed-batch fermentation was initiated using a script to trigger the feed pump to the DO spike.

Throughout the fermentation, we took periodic samples for OD measurements, CWW determination and pDNA extraction.

Microscopic evaluation

Usually, *E. coli* cells are rod shaped and approximately 800 nm wide and 2.5 µm long. However, the strain used in this study showed a filamentous morphology where some cells elongate significantly as shown in the example in Figure 5. Previous publications have noted that pDNA production strains of *E. coli* commonly show variations in morphology [5]. Additional studies have demonstrated that cell wall synthesis genes, involved in cell division, have a lower expression profile in host cells producing pDNA [8]. This deficit leads to incomplete separation of the cells during a cell cycle and therefore the elongated shape.

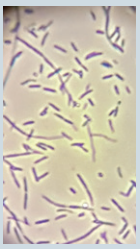


Fig. 5: Microscopic view of *E.coli* DH5α (DMSZ 6897) after growth for 23 h at 37 °C in a bioreactor.
An objective with 100 x magnification was used.

Bioprocess trends

Batch fermentation

Three hours following inoculation, the DO (Figure 6, blue curve) stabilized at a level of 30 %. This value was maintained over the course of fermentation.

Once the hourly sampling was initiated, (13 h inoculation time) drops in DO measurement were visible, caused by sampling process.

Before drawing the sample, medium remaining in the sample port was pushed back to get a well-mixed and representative sample. This pushing back step seems to cause the drops in DO.

The batch fermentation was started with a glucose concentration of 6.6 g glucose per 150 mL medium (Table 6). At 17 h of inoculation time, a sharp DO-spike reaching 50 % was visible, marking the consumption of the supplied glucose.

We ended the batch fermentation together with the fed-batch fermentation after 23 h.

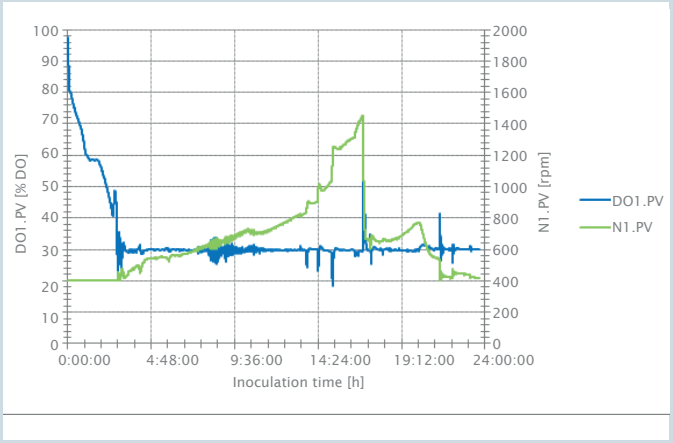


Fig. 6: Process graphs of the batch fermentation of *E. coli* DH5α using the DASbox Mini Bioreactor System. The trend of the dissolved oxygen is shown by the blue curve (DO.PV). The green curve (N.PV) shows the trend of agitation to control the DO value.

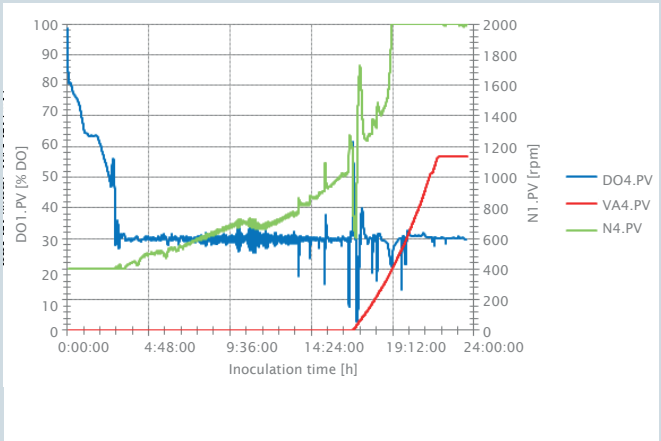


Fig. 7: Process graphs of the fed-batch fermentation of *E. coli* DH5α using the DASbox Mini Bioreactor System. The trend of dissolved oxygen is shown by the blue curve (DO.PV).The green curve (N.PV) shows the corresponding agitation speed required to control the DO. Total feed volume (VA.PV) is shown by the red curve.

Fed-batch fermentation

The DO curve (Figure 7, blue) shows a trend comparable to the batch fermentation. Again after the 13 h inoculation time, drops in DO are visible once a sample is withdrawn.

As the batch process, the fed-batch process was started with a concentration of 6.6 g glucose per 150 mL medium. As in the batch process described before, at 17 h after inoculation a sharp DO spike reaching 60 % DO was visible marking the consumption of the initially supplied glucose. The DO spike marked the end of the batch phase and triggered the feed start. The feed profile is summarized in Table 12.

As the feed rate increases (red curve) the stirrer speed (green curve) reached the maximum of 2000 rpm causing the DO control to increase gas flow.

Growth curves

E. coli DH5α growth curves (Figure 8) show a comparable growth during the batch phase of the processes (until t=17 h). The growth continued in both fermentation runs until t=18 h. At that point, batch fermentation showed a decline in optical density whereas the fed-batch fermentation continued to grow until a stationary phase was reached (t=21).

CWW measurements (data not shown) followed the trend of the optical density measurements. For batch fermentation a maximal CWW of over 70 g/L is reached at 17 h inoculation time. For fed-batch the maximum of 110 g/L is reached 23 h after inoculation.

Plasmid DNA yield

After a continuing period of plasmid production, both processes show a peak at 18 h inoculation time. For the batch process (Figure 9, blue curve) this is again followed by a steady phase and finally a decrease of plasmid yield after 21 h inoculation time. However, the fed-batch process (Figure 7, red curve) is reaching a second peak at 20 h inoculation time. The decrease in the pDNA yield in the fed-batch process after 21 hours may be caused by degradation of the plasmid. However, additional experiments are required to verify this hypothesis.

The maximum pDNA yields per reactor were 28.7 mg in the batch process and 56.5 mg in the fed-batch process.

Taking the increased optical density into account (Figure 8), these data confirm that the increase in pDNA correlates well with the increased number of cells. Thus, the optimization of cell density is the critical factor for realizing a more efficient production process.

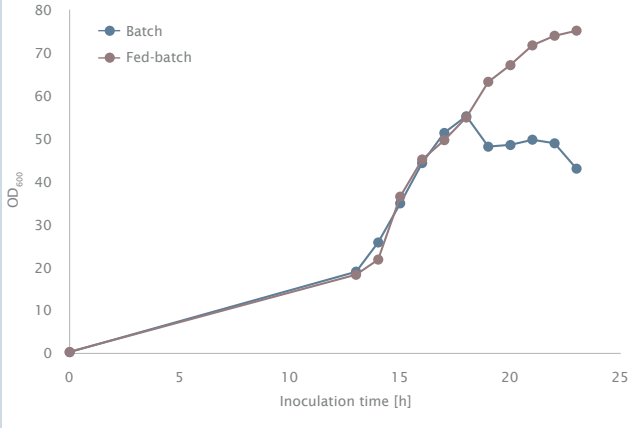


Fig. 8: Optical density measurements of batch and fed-batch fermentation.

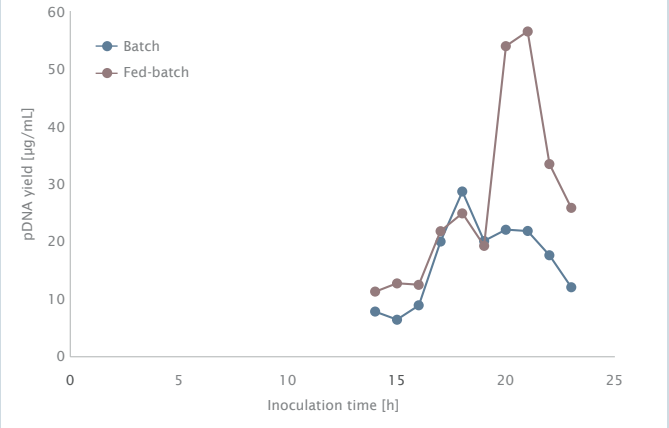


Fig. 9: Comparison of the pDNA yield of batch and fed-batch fermentation.

Conclusion

We successfully designed and executed a fermentation protocol of *E. coli* DH5α demonstrating plasmid DNA production in the Eppendorf DASbox Mini Bioreactor System.
We compared plasmid production yield in a batch and a fed-batch fermentation and analyzed the optimal harvest time point for each case:

In the batch process, we have shown that the yield of pDNA per reactor is decreasing over time after the batch phase is finished (DO-spike). Thus, for a batch process, the DO spike could serve as indicator for the optimal harvest time point. However, additional experiments are needed for verification.

In the fed-batch process we observed a much higher optical density as well as increased yield of plasmid DNA compared to the batch process. In a late phase of the fed-batch process the plasmid yield is decreasing, indicating that also in the fed-batch process the harvest time point needs to be further optimized.

In both the batch and fed-batch process we confirmed a correlation between plasmid production yield and cell growth. One can conclude, that plasmid production yield (as measured by optical density) can be further increased by scaling up the fermentation and optimizing for high cell density. We have demonstrated high cell density during *E. coli* fermentation scale- up in 100 L pilot scale BioFlo® 610 fermenters previously [9].

Literature

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Ordering information

Description	Order no.
DASbox® Mini Bioreactor System, for microbial applications, max. 25 sL/h gassing, 4-fold system	76DX04MB
DASware® control, including PC, OS, and licenses, for 4-fold DASbox® Mini Bioreactor System	76DXCS4
DASbox® Vessel , 60 – 250 mL, overhead drive, 2 Rushton-type impellers	SR0250ODLS
New Brunswick Innova® 42R, refrigerated, stackable up to 2-fold, 230 V/50 Hz, orbit 2.5 cm (1 in)	M1335-0016
Eppendorf BioPhotometer® D30 , 100 – 240 V ±10 %/50 – 60 Hz (EU)	6133000001
Centrifuge 5427 R , rotary knobs, refrigerated, without rotor, 230 V/50 – 60 Hz (EU)	5409000012
Eppendorf Safe-Lock Tubes, 2.0 mL, Eppendorf Quality, colorless, 1,000 tubes	0030120.094

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