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Improvement of Plasmid Volumetric Yield by Addition of Glycerol and Phosphate Buffer in *Escherichia coli* TOP10 Batch Culture

Anindyajati[1], Salma Aulia Afifah[1], Catur Riani[1], Marselina Irasonia Tan[2], Dessy Natalia[3], Ernawati Arifin Giri-Rachman[2], Anita Artarini[1]*

[1]*School of Pharmacy, Institut Teknologi Bandung, Bandung 40132, Indonesia*
[2]*School of Life Sciences and Technology, Institut Teknologi Bandung, Bandung 40132, Indonesia*
[3]*Faculty of Mathematics and Natural Sciences, Institut Teknologi Bandung, Bandung 40132, Indonesia*

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ABSTRACT

The investigation of mRNA development has gained substantial interest, particularly in the *ex vivo* and *in vivo* therapy. mRNA is widely used for the development of gene editing-based therapies and mRNA vaccines. The aim of this study was to optimize the medium and harvest time to increase plasmid DNA production as part of mRNA production. This study modified used a medium modification approach to achieve high density culture of *Escherichia coli* TOP10 pGEMT-N in batch cultivation method. Various media formulations were assessed, including LB; LB with phosphate buffer (K_2HPO_4 12.549 g/L and KH_2PO_4 2.31 g/L); LB with glycerol (50 g/L); LB with glycerol and phosphate buffer; LB with phosphate buffer, glycerol, glucose (15 g/L), and galactose (15 g/L). The effect of additional carbon sources and phosphate buffer on culture density was measured through OD_{600} and wet cell weight analysis. The highest OD_{600} and wet cell weight was observed when LB with glycerol and phosphate buffer was used, with OD_{600} of 4.78±0.14 and wet cell weight of 36.00±0.63 mg/ml. Plasmid DNA was subsequently isolated from these cultures following 5- and 7.5-hour incubation periods. The utilization of LB medium with glycerol and phosphate buffer resulted in a substantial increase in the volumetric concentration of plasmid DNA of 1,516.97±385.00 ng/ml after 5 hours of incubation. In conclusion, a remarkable enhancement in plasmid DNA volumetric yield within 5 hours was achieved by addition of glycerol and phosphate buffer to LB medium, leading to incubation period.

1. Introduction

Plasmid DNA (pDNA) is very important for modern biotechnology. pDNA is needed in large quantities to be used in many ways. pDNA is widely used for transfection or as a basis for developing promising vaccines in the future because it allows efficient immunization (Kis *et al.* 2020). After pDNA, the development of mRNA is increasingly interesting to study, especially for *ex vivo* and *in vivo* therapy.

mRNA is a type of single-stranded ribonucleic acid transcribed from DNA strands, which carries the code for protein synthesis and can then be translated into functional proteins. Unlike DNA-based products, mRNA transcripts have relatively high transfection efficiency and low toxicity because the mRNA does not need to enter the nucleus to function. mRNA has no potential risk of insertion mutagenesis and oncogenesis(Qin *et al.* 2022). mRNA expression that is transient, has high delivery efficiency *in vivo*, and has high efficiency in gene editing is widely used for genome editing such as CRISPR/Cas9, ZFNs, and TALENs (Zhang *et al.* 2019).

Besides being developed for gene therapy and gene editing, mRNA is widely used for vaccine development. The COVID-19 pandemic created a demand for rapid, high-volume production of vaccines. One of the new technologies and approaches being developed is the mRNA vaccine. mRNA vaccines serve the same purpose as traditional vaccines but work in a slightly different way. In traditional vaccines, parts or intact components of attenuated viruses or bacteria are injected, while mRNA vaccines use parts of the viral

* Corresponding Author
E-mail Address: anita@itb.ac.id

or bacterial genes to stimulate an immune response (Liu 2019).

The mRNA vaccine platform technology holds great promise for facilitating immunization against SARS CoV-2 (Kis *et al.* 2020), an example of which is the vaccine developed by Pfizer-BioNTech and Moderna. mRNA vaccines make it possible to carry the genetic material directly into the cell, thereby enabling the expression of endogenous proteins. Another advantage of mRNA-based vaccines is that they can be produced without cell culture by *in vitro* transcription, thereby reducing issues regarding quality and safety. Its production without cell culture allows for a simpler, faster, and more economical purification process compared to other vaccine platforms (Machado *et al.* 2021).

The *in vitro* transcription process starts with linearizing the DNA template, performing *in vitro* transcription, then removing the rest of the template using DNase for its purification. The mRNA platform technology is a new technology which needs to be developed in terms of production to get better results effectively and efficiently. This mRNA is needed in large quantities to be used, one example is for one dose of mRNA vaccine, 12-100 µg of mRNA is needed (Jackson *et al.* 2020). In the in vitro transcription process, one potential method to obtain a substantial quantity of mRNA is by increasing the production of pDNA acting as the template.

Enhancing plasmid production can be achieved by increasing the volumetric yield, specific yield, or both (Gonçalves *et al.* 2014a). Factors that can affect specific yields are the *Escherichia coli* host strain used (Gonçalves *et al.* 2014b), genetic modification of the host strain (Borja *et al.* 2012), the media used (Gonçalves *et al.* 2014a), and the type of and the size of the plasmid used (Galindo *et al.* 2016). Factors that can affect the volumetric yield of plasmids are the conditions for growing *E. coli*, such as pH, temperature, dissolved oxygen, media used, and cultivation methods to obtain high biomass (Islas-Lugo *et al.* 2016).

This study focuses on medium optimization to attain a high-density culture (HDC) for *Escherichia coli* TOP10 pGEMT-N. To achieve this, LB media was modified by adding alternative carbon sources while also implementing buffering strategies. We also determined the optimal harvest time that would yield the highest quantity of pDNA. The optimum medium composition and incubation time acquired will provide both the optimal condition for cultivating *E. coli* TOP10 pGEMT-N serving as the source of pGEMT-N for in vitro transcription templates and also shed light t on the impact of carbon sources and buffered media on the growth dynamics and plasmid content within high-density *E. coli* cultures.

2. Materials and Methods

2.1. Bacteria, Plasmid, and Media Composition

The bacteria used in this study was recombinant *Escherichia coli* TOP10 carrying plasmid pGEMT-N (*E. coli* TOP10 pGEMT-N), which was available at the Laboratory of Pharmaceutical Biotechnology, School of Pharmacy, Institut Teknologi Bandung. pGEMT-N plasmid was constructed by cloning the SARS-CoV-2 N gene into pGEMT (Promega), with the size of 4,276 bp.

In this study, media components used were as follows: NaCl (Merck), tryptone (Oxoid), yeast extract (Oxoid), agar (Oxoid), K_2HPO_4 (Merck), KH_2PO_4 (Merck), glycerol (Merck), glucose (Merck), galactose (Merck), and ampicillin antibiotics (Bernofarm). Luria-Bertani (LB) agar medium comprised 1% NaCl, 1% tryptone, 0.5% yeast extract, and 1.5% agar.

2.2. Plasmid Characterization and Confirmation

2.2.1. Plasmid Isolation

Recombinant *E. coli*, TOP10 pGEMT-N bacteria, were grown from glycerol stock on an LB agar medium containing 100 µg/ml ampicillin and then incubated for 18 hours at 37°C. Overnight cultures were made in an LB medium with a minimum ratio of media volume over tube size of 1:5 and an incubation time of 18-20 hours at 37°C and 150 rpm. Plasmids were isolated using PrestoTM Mini Plasmid Kit (Geneaid). First, cells were harvested by centrifugation at 16,000 ×g for 1 minute. The cell pellet was separated from the medium, resuspended using PD1 buffer, and lysed using PD2 buffer. The neutralization process was carried out using PD3 buffer and centrifuged at 16,000 ×g for 3 minutes. The DNA was bound to the PD column, washed using W1 buffer and washing buffer, and centrifuged at 16,000 ×g for 30 seconds. The DNA was eluted with nuclease-free water and then collected in a microcentrifuge tube.

2.2.2. Plasmid Concentration and Purity Analysis

The concentration of the isolated plasmids was measured using µDropTM Plate in Multiskan GO spectrophotometer (Thermo Scientific). As much as 1 µL sample was used to determine the concentration, and nuclease-free water was used as a blank. The

readings were done at 260 nm (A_{260}) and 280 nm (A_{280}) wavelengths. The result obtained was in the form of absorbance, and then the plasmid concentration was calculated using the following equation, while the ratio of absorbance at 260 and 280 readings was used to assess DNA purity.

Plasmid concentration (ng/µL) = A260 × 50 × (10 / 0.52)

2.2.3. Plasmid Migration Analysis

Migration analysis was performed by 0.8% agarose gel electrophoresis. pcDNA3.1-Zeo(+), a size of 5,015 bp, was used as a control. The amount of plasmid used was 200 ng, whose volume was adjusted according to the plasmid concentration acquired from the A_{260} reading. DNA electrophoresis was carried out at 100 V for 30 minutes. Following electrophoresis, the gel was stained with an ethidium bromide solution for 5 minutes and de-stained with sterile distilled water for 10 minutes. Visualization was carried out by exposing the gel to 254 nm UV light.

2.2.4. Plasmid Restriction Analysis

Restriction analysis was performed using NcoI (ThermoScientific) and SacI (ThermoScientific) restriction enzymes. The restriction reaction composition was as follows: 500 ng plasmid, 0.2 µL of 10 U/µL enzyme, 2 µL of 10× buffer, and nuclease-free water until the reaction volume reached 20 µL. The mixture was incubated at 37°C for 3 hours. A 1% agarose gel electrophoresis was carried out at 100 V for 25 minutes, followed by ethidium bromide staining and observation under 254 nm UV light.

2.3. Growth Curve of *E. coli* TOP10 pGEMT-N Determination

E. coli TOP10 pGEMT-N was grown in 10 ml of LB medium with 100 µg/ml ampicillin in a 50 ml tube and incubated at 37°C, 150 rpm for 18-20 hours. The overnight culture was diluted to obtain an optical density of 0.2 at 600 nm wavelength (OD_{600}). The diluted culture was grown at 5% (v/v) in 200 ml LB medium with 100 µg/ml ampicillin in 1L Erlenmeyer flask. OD_{600} and wet cell weights were measured at 0; 2,5; 5; 7,5; 24, and 26 hours. Samples were taken from the culture for OD_{600} and wet cell weight measurements. Wet cell weight was measured by sampling 5 ml of the culture and centrifugation for 2 minutes at 16,000 ×g. The supernatant was discarded. After ensuring no supernatant was left in the cell pellet, the wet cell weight was weighed using a milligram scale. The results of optical density reading and wet cell weight at each sampling point were plotted into growth curves to see the growth profile of *E. coli* TOP10 pGEMT-N. The growth curve obtained will later be compared to the culture grown in various media compositions. Five different medium compositions were used in this study (Table 1). The components were LB with the composition of 1% NaCl, 1% tryptone, and 0.5% yeast extract; phosphate buffer with the composition of 12.549 g/L K_2HPO_4 (Merck) and 2.31 g/L KH_2PO_4 (Merck); and carbon sources in the form of 50 g/L glycerol, 15 g/L glucose, and 15 g/L galactose. Ampicillin antibiotics with a concentration of 100 µg/ml were added to each medium composition. Observation of the growth profile of *E. coli* TOP10 pGEMT-N was carried out on LB medium with the addition of phosphate buffer; LB with the addition of glycerol; LB with the addition of phosphate buffer and glycerol; and LB with the addition of phosphate buffer, glycerol, glucose, and galactose (Table 1). Sampling was conducted at 0; 2.5; 5; 7.5; 24; and 26.5 hours. Samples taken were processed for OD_{600} value and wet cell weight measurements. The optical density and cell weights at each sampling point were made into growth curves and then compared with the growth profile on the LB medium.

2.4. Plasmid DNA Quantification

Plasmid of cell pellet from sampling at 5- and 7.5-hour incubation time on all medium compositions were isolated by equating the cell weights to 15

Table 1. Variation of medium composition used in this study

Medium	Composition	Concentration (g/L)
*LB	NaCl	10
	Tryptone	10
	Yeast extract	5
LB with the addition of phosphate buffer	LB	*
	K_2HPO_4	12.549
	KH_2PO_4	2.31
LB with the addition of glycerol	LB	*
	Glycerol	50
LB with the addition of phosphate buffer and glycerol	LB	*
	K_2HPO_4	12.549
	KH_2PO_4	2.31
	Glycerol	50
LB with the addition of phosphate buffer, glycerol, glucose, and galactose	LB	*
	K_2HPO_4	12.549
	KH_2PO_4	2.31
	Glycerol	50
	Glucose	15
	Galactose	15

*follows the LB medium composition

mg. Plasmid concentration was measured and calculated using a µDrop™ Plate in MultiskanGO spectrophotometer (Thermo Scientific) as mentioned above. Plasmid concentration was also carried out semi-quantitatively using agarose gel electrophoresis. The intensity of each plasmid band in the electrophoregram was compared against standard markers whose concentrations were known using ImageJ software. Plasmid quantification was also carried out using quantitative PCR (qPCR) method using the SensiFast Probe No-ROX One-Step Kit (Bioline) with probe nCoV_N1 (5'-FAM-ACCCCGCATTACGTTTGGTGGACC-BHq1-3') and primer pair targeting N gene (2019-nCoV_N1_F (5'-GACCCCAAAATCAGCGAAAT-3') and 2019-nCoV_N1_R (5'-TCTGGTTACTGCCAGTTGAATCTG-3') (Integrated DNA Technologies)), giving a product size of 72 bp. Positive control 2019-nCoV_N (Integrated DNA Technologies) was used to calculate the plasmid copy number of pGEMT-N. The qPCR reaction consisted of 5 µL of 0.1 ng/ µL DNA template, 0.8 µL of 10 µM forward and reverse primer each, 0.2 µL of 10 µM probe, and PCR water giving the final volume of 20 µL. The DNA amplification conditions included 1 cycle of DNA polymerase activation at 95°C for 2 minutes followed by 45 cycles of denaturation at 90°C for 5 seconds and annealing and elongation at 60°C for 20 seconds. The positive control was prepared at 10^5-10^2 copy number dilution series, while the negative control was non-template control (NTC).

3. Results

3.1. Characterization of pGEMT-N Plasmid

The culture model used in this study was *E. coli* TOP10 pGEMT-N. Initially, the plasmid was isolated and then confirmed using agarose gel electrophoresis. The analysis of undigested plasmid showed that the pGEMT-N, with size of 4,276 bp, migrated further than the pcDNA3.1-Zeo(+), with size of 5,015 bp (Figure 1A). Restriction analysis of pGEMT-N using NcoI and SacI showed two bands with size of 2,909 bp and 1,384 bp, which corresponds to the theoretical size of the plasmid (Figure 1B). These results confirmed the characteristics of pGEMT-N, which was used for the next experiments.

3.2. Effect of Culture Media Modification on *E. coli* TOP10 pGEMT-N Growth

One way to increase plasmid DNA production was to produce high cell density. One of the strategies to achieve high cell density was by modifying the media composition during the culture process. In this study, we compared five different media compositions and observed the cell growth through the measurement of OD_{600} and determination of wet cell weight. The result showed that the stationary phase of *E. coli* TOP10 pGEMT-N on LB medium was observed at 5 hours. In the other four media, the logarithmic phase was observed to be longer, and the stationary phase was achieved after 7.5 hours (Figure 2A). The culture medium giving the highest OD_{600} value of 4.78±0.14 was LB with the addition of glycerol and phosphate buffer. The OD_{600} value has increased 3.6 times compared to LB, which was 1.31±0.15. When the medium was supplemented with buffer, glycerol, glucose, and galactose, there was a decrease in OD_{600} to 3.9±0.08 (Figure 2A). In line with OD_{600}, the growth profile of *E. coli* TOP10 pGEMT-N based on wet cell weight resulted in the highest cell weight in LB medium with the addition of glycerol and buffer, which was 9.36±0.63 mg/ml. This value was 2.7 times higher than the LB medium, which was 3.44±0.34 mg/ml (Figure 2B).

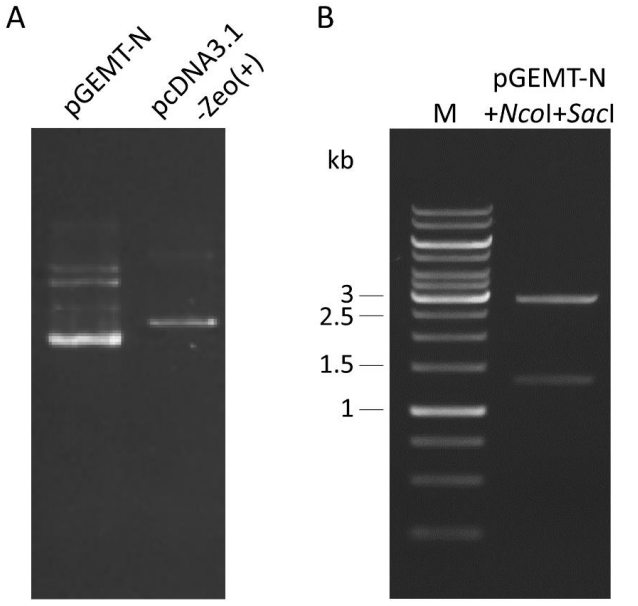

Figure 1. Characterization of pGEMT-N plasmid. (A) Circular pGEMT-N was compared with pcDNA3.1-Zeo(+) using agarose gel electrophoresis. (B) Restriction analysis of pGEMT-N using NcoI and SacI, M = 1 kb DNA ladder

3.3. Effect of Cell Culture Media on pGEMT-N Yield

We further analyzed the plasmid yield from samples obtained at 5 and 7.5 hours of incubation. The measurement of plasmid yield was performed by

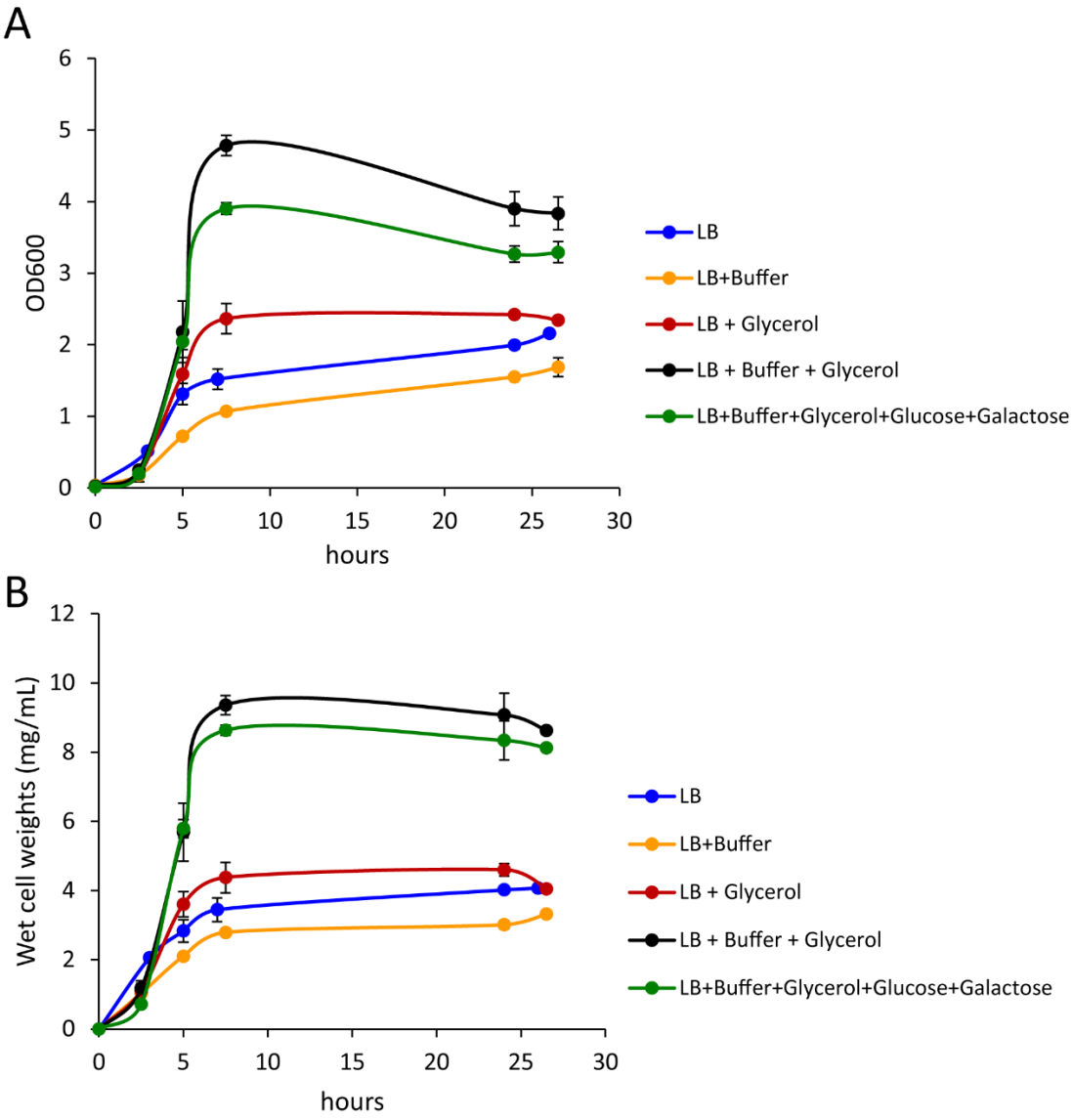

Figure 2. Growth profile of *E. coli* TOP10 pGEMT-N measured by OD_{600} (A) and wet cell weight (B). Cultivation conditions were 37°C with an agitation speed of 230 rpm. Data were obtained from three independent experiments and expressed as mean ± SEM

measuring A_{260}, using semi-quantitative calculation from agarose gel electrophoresis, and using qPCR. The plasmid yield was expressed as specific yield and volumetric yield. The specific yield was calculated from the number of plasmids per unit cell mass. The results showed no pattern of increase in plasmid-specific yield. The highest specific yield of 8,986.6±62.2 ng plasmid/mg cells was obtained from bacteria cultured in LB medium with phosphate buffer, followed by LB medium, LB with added glycerol, and LB with added buffer, glycerol, glucose, and galactose. The lowest value was obtained from culture in LB with the addition of glycerol and buffer (Figure 3A). Based on the A_{260} measurement, plasmid production at 7.5 hours was higher when compared to plasmid production at 5 hours. However, when confirmation was carried out using agarose gel electrophoresis and qPCR, the plasmid-specific yields between 5- and 7.5-hours incubation time were not different (Figure 3A). Based on the electrophoresis and qPCR result addition of buffer, glycerol, glucose, and galactose to the LB medium resulted in the decrease of plasmid specific yield (Figure 3A).

The plasmid volumetric yield was calculated from the number of plasmids per unit culture volume. Based on the results of A_{260} measurement, the volumetric

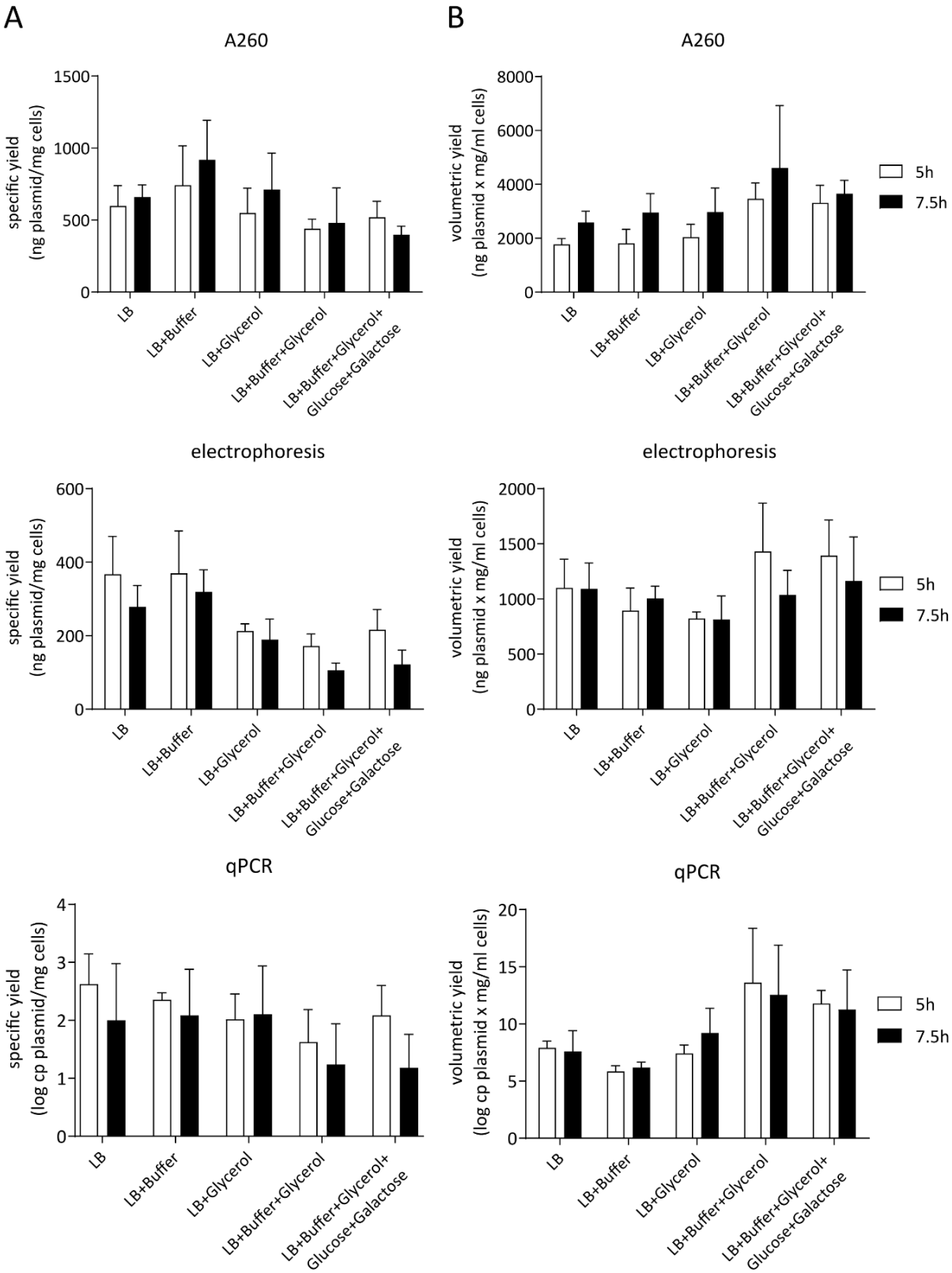

Figure 3. Calculation of plasmid specific yield (A) and volumetric yield (B) at 5 and 7.5 hours. The plasmid was quantified using A_{260} measurement, semi-quantitative calculation based on agarose gel electrophoresis results, and qPCR. The plasmid specific yield was calculated by dividing the plasmid amount to the wet cell weight, meanwhile the plasmid volumetric yield was calculated by multiplying plasmid amount to the wet cell weight per ml culture. Data were shown as mean ± SEM from at least 3 independent experiments

yield increased with the addition of buffer and carbon sources. The highest volumetric yield was obtained from culture in LB medium with the addition of buffer and glycerol, with a concentration of 5,309±2,213.42 ng/ml, followed by LB medium with the addition of buffer, glycerol, glucose, and galactose; LB with the addition of glycerol; LB; and LB with the addition of buffer (Figure 3B). The same pattern of increase was observed when the plasmid yield was measured by qPCR and measured semi-quantitatively using agarose gel electrophoresis. The culture giving the highest volumetric yield was grown in LB with the addition of buffer and glycerol, with a concentration of 1,516.97±385.00 ng/ml (Figure 3B).

4. Discussion

To achieve a high-density culture, a balanced medium provides nutrients needed in sufficient quantities as a source of energy, biomass formation, and cell growth. In general, growth media contain sources of carbon and nitrogen, various salts, and trace elements. Medium selection is essential for the fermentation process because it directly affects product concentration, yield, and volumetric productivity (Kampen 2014). In this study, additional carbon sources and buffers were added, meanwhile the nitrogen source in the LB medium was provided from tryptone and yeast extract. The main carbon source in the LB medium was only available in the form of oligopeptides in yeast extract, where the concentration cannot be clearly determined. The preferred carbon sources for *E. coli* are glucose, glycerol, and lactose, which can support faster growth rates (Bren *et al.* 2016). As additional carbon sources are added, the cell metabolic processes will increase and produce metabolites that can lower the overall culture pH and inhibit cell growth itself, so the addition of buffers is used to prevent pH fluctuations that may occur. Another additional nitrogen source can also be added in the form of ammonium chloride, however this study focused initially on adding a carbon source and buffer.

Based on the growth curve obtained in this study, there was a difference in the logarithmic phase time between LB and other media because the medium with modified composition contains components that can extend the log phase. Buffers could reduce pH fluctuations during the cultivation process, and various carbon sources such as glycerol, glucose, and galactose are the primary energy sources for growing cells (Sohoni *et al.* 2015). In line with OD_{600} data, the growth profile of *E. coli* TOP10 pGEMT-N based on wet cell weight resulted in the highest cell weight in the LB medium with the addition of glycerol and buffer.

In a previously reported study, glucose and ammonium chloride were used as the primary carbon and nitrogen sources in the medium with phosphate buffer. This formulation successfully increased the OD_{600} value of *E. coli* DH5α to 3.5 times higher than LB Lennox, with the highest OD_{600} value obtained at 27 hours incubation time (Ng 2021). The addition of phosphate buffer to that medium showed a pH decrease in the buffered culture, followed by a gradual increase to 7.6 at the end of the log phase. The result did not significantly change compared to the non-buffered medium, which decreased from 7.2 to 5.6, followed by an increase to 8.9 at the end of the log phase (Ng 2021). Another study modified the medium for protein production in *E. coli* BL21(DE3) to achieve a high-density culture. The cultivation method was a fed-batch method, and the medium was composed of yeast extract and glycerol with an optimal ratio of 1.5-1.6. This medium increased the OD_{600} value by 3.7 times compared to the LB medium at 6 hours incubation time (Ganjave *et al.* 2022).

It was known that adding buffer and carbon sources did not increase the specific yield of plasmids but increased the volumetric yield. The addition of buffer and carbon source was known to support cell growth, while the building blocks for the formation of plasmid DNA remain unchanged. In previous studies, the effect of the medium was observed on the production of large plasmids. *E. coli* DH5α carrying plasmids of different sizes was grown on various media. Plasmids production from culture in the Terrific Broth (TB) medium provided a higher specific yield when compared to the LB medium (Galindo *et al.* 2016). TB medium contains higher yeast extract than LB, thus it provides higher levels of asparagine, glutamate, glycine, histidine, and leucine. These components are needed for DNA synthesis (Mairhofer and Lara 2014). The availability of aromatic amino acids (tyrosine, phenylalanine, and tryptophan) in the culture medium dramatically affects the yield of pDNA (Passarinha 2021).

The effect of carbon source addition on plasmid production has been studied previously. The addition of glycerol with different concentrations was known to increase the specific yield of plasmids with the

most optimal glycerol concentration of 50 g/L (Islas-Lugo *et al.* 2016). The addition of glycerol prevents the repression of intermediate metabolites and the accumulation of inhibitive organic acids to a certain extent. Therefore, the addition of glycerol affected plasmid DNA production and *E. coli* cell growth (Xu *et al.* 2005). However, the addition of glycerol as a carbon source was not the only parameter that improved the volumetric and specific yield of plasmid. The use of NH_4OH for pH control in the previous study could serve as an additional nitrogen source besides yeast extract (Islas-Lugo *et al.* 2016). This approach could be further explored for the pGEMT-N plasmid production.

In both the specific yield and the volumetric yield, the results of the OD_{260} measurements were observed to have a more significant amount than the electrophoresis results analysis. This difference occurred because the product of plasmid isolation might contain contaminants that can be detected at a wavelength of 260 nm. The A260 measured all nucleic acids in the sample, especially chromosomal DNA, degraded plasmid DNA (Stepanov and Nyborg 2003), and other unknown contaminants. An analysis of these contaminants needs to be carried out to determine whether these contaminants can affect the yield of mRNA after the *in vitro* transcription process. The plasmid yield on the electrophoretic calculation results had a higher concentration at the 5th hour than at the 7.5th hour. This result was obtained because, over time, the number of cells will increase, supported by the addition of buffer and carbon sources, but the nutrients needed to form plasmids will decrease. For further research, nutritional sources supporting plasmid formation can be added, such as additional nitrogen sources, trace elements, or increasing the yeast extract concentration to increase the specific yield of plasmids.

The production of pDNA on a laboratory scale was generally carried out in an Erlenmeyer. However, the Erlenmeyer was a simple and inexpensive bioreactor, several limiting factors exist, such as the lack of process control and monitoring. Medium optimization could increase the production and quality of pDNA isolated from a culture in Erlenmeyer. However, other problems need to be developed for solutions, for example, the relatively low capacity and oxygen transfer in Erlenmeyer which can limit the amount of pDNA production (Galindo *et al.* 2016). In addition to oxygen demand, pH was also an important parameter. Although buffer has been added to the medium, it was necessary to monitor and control the pH in real-time and add a pH regulator as a base to keep the pH at an optimal point for pDNA production. For further research, these parameters could be optimized using methods that can be carried out on a laboratory scale to obtain a much higher yield of cells and plasmids.

Conflict of Interest

The authors declared no conflict of interest.

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