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SHORT COMMUNICATION

Improved production of large plasmid DNA by enzyme-controlled glucose release

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Abstract The laboratory-scale production of plasmid DNA (pDNA) is hindered by the limitations of shake flasks, such as mass transfer capacity and lack of pH control. Consequently, better schemes for pDNA production in shake flasks are needed. pDNA production can be improved by increasing the amount of biomass, increasing the pDNA yield on biomass ($Y_{pDNA/OD}$), or both. In this study, we characterized the production of three differently sized plasmids (5.4, 6.0, and 7.8 kbp, respectively) cultured in Lysogenic Broth (LB), Terrific Broth (TB), and EnPresso B Plasmid (EBP) culture medium that releases glucose to the broth enzymatically. Higher cell densities, higher acetate accumulation, and higher pDNA titers, were obtained in cells cultured in TB than in those cultured in LB medium. The enzyme-controlled glucose release system resulted in an important increase in cell densities, while the $Y_{pDNA/OD}$ increased up to threefold. The most important increase in pDNA titer was observed for the 7.8-kbp plasmid, which raised from 1.6 to 4.3 mg/L in LB and TB medium, respectively, to 26.6 mg/L using the EBP medium. These results show that controlled substrate delivery is useful to increase the production of large-sized pDNA in shake flasks.

Keywords DNA vaccines · Glucose release · Growth control · Plasmid DNA production · Shake flask culture

The use of plasmids in modern biotechnology is of prime importance. Relatively large amounts (hundreds of milligrams) of plasmid DNA (pDNA) are required for applications such as transfection and the development of DNA vaccines (Lara et al. 2012; Mairhofer and Lara 2014). The laboratory-scale production of pDNA is commonly carried out in shake flasks. Although shake flasks are simple, inexpensive, and widely available bioreactors, there are several limiting factors when elevated amounts of pDNA are to be produced, such as a general lack of process control and monitoring. Careful optimization of medium components can increase pDNA production and quality in shake flasks (Martins et al. 2015), however, other issues also need to be resolved. For example, the relatively low oxygen transfer capacity of shake flasks (Büchs 2001) limits the amount of pDNA that can be produced. The oxygen demand of *Escherichia coli* cultures for pDNA production can be very high (Lara et al. 2011); consequently, it is challenging to provide and maintain fully aerobic conditions in shake flask cultures if high cell densities are to be attained. The design of plasmids may require the insertion of more than one sequence of interest, thus increasing the size of the plasmid. It has been reported that as the size of the plasmid increases, the biomass yield and cell viability, as well as plasmid copy number decreased (Cheah et al. 1987) (Smith and Bidochka 1988). Moreover, Khosravi et al. (1990) reported a significant increase of the oxygen demand of *E. coli* when the size of the plasmid increased. Therefore, although larger plasmids may be required

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for biotechnology applications, the production of such relatively larger plasmids in shake flasks is less efficient than that of shorter ones. In an instrumented bioreactor, oxygen consumption can be controlled by adding the carbon source to the medium in a controlled manner, an approach which also decreases the growth rate and prevents overflow metabolism (Mairhofer and Lara 2014). This strategy is rarely implemented in a shake flask. However, it has been shown that the carbon source (glucose) can be added to the medium as a polymer that is non-degradable by *E. coli*. Glucose is released to the broth by action of a glucoamylase (Krause et al. 2010). This approach mimics a fed-batch scheme; the cell densities obtained are much higher than those obtained using common culture media, and the growth rate and, consequently, the oxygen demand are reduced. This strategy has been proven to be a very efficient way to improve recombinant protein production in shake flasks (Jeude et al. 2006; Krause et al. 2010).

We have studied the efficiency of EBP medium combined with enzyme-controlled glucose release for the production of three differently sized plasmids (5.4, 6.0, and 7.8 kbp, respectively) which share a common backbone. The results were compared to those obtained using traditional media, such as Lysogenic Broth (LB) and Terrific Broth (TB).

Escherichia coli strain DH5α [φ80*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA*1*endA*1 *hsdR*17(rk−, mk+) *gal- phoA supE44* λ- *thi*-1 *gyrA96 relA1*] was used in all the experiments. Three different transformants were obtained by the electroporation of competent cells with the three differently sized plasmids. Plasmid pcDNA3.1(+) (5.4 kbp; Invitrogen/Thermo Fisher Scientific Corp., Carlsbad, CA) contains selectable markers and genetic elements for the expression of a cloned gene in mammalian cells, as well as an ampicillin resistance gene and pUC replicon for maintenance in *E. coli*. Plasmid pHN (6.0 kbp) was constructed from the pcDNA3.1(+) plasmid by inserting a viral hemagglutinin neuraminidase gene under transcriptional control of the cytomegalovirus promoter. The pHN plasmid is being evaluated as a DNA vaccine against mumps in humans (Herrera et al. 2010). Plasmid pGH (7.8 kbp) was constructed by cloning a sequence coding for the gH glycoprotein of Herpes simplex 1 virus in pcDNA3.1(−) (Invitrogen/Thermo Fisher Scientific Corp.); it has the same characteristics as pcDNA3.1(+), except that reverse cloning sites are provided. The transformed strains were cultured in LB medium supplemented with ampicillin disodium salt (100 mg/L) to the late exponential growth phase and conserved in glycerol (40 % v/v) at – 80 °C. A small amount of the cryo-preserved strains was plated on solid (2 % agar) LB medium supplemented with ampicillin disodium salt (100 mg/L) for 30 h at 37 °C, following which time the cells were taken from the LB agar plates and transferred to liquid LB medium (10 mL in 50-mL tubes). Pre-cultures were developed by incubating the inclined (30–45°) tubes at 37 °C and under agitation of 150 rpm for 4–6 h. These cultures were used to inoculate (2 mL of pre-culture per shake flask) the main culture shake flask. For cultures in LB and TB media, 250-mL baffled Erlenmeyer flasks were used. Each flask was filled with 50 mL of the medium supplemented with ampicillin disodium salt (100 mg/L) and capped with a sponge closure. The EnPresso B Plasmid (EBP) medium (BioSilta Oy, Oulu, Finland; product number B13001) has been specifically designed for plasmid production. The product is composed of tablets containing the components of the medium: mineral salts, phosphate buffer, complex nutrients, and a soluble glucose polysaccharide. Glucose is released from the polysaccharide into the broth by an enzymatic reaction mediated by an enzyme provided in the kit. We followed the protocol indicated by the manufacturer, as follows: first, two tablets of the medium were dissolved in 50 mL of sterile water contained in 500-mL standard shake flasks; second, Reagent A (33 μL of enzyme solution, yielding a final concentration of 2 U/L) and cells were added. Sponge closures were used in all cases. All cultures were carried out at an agitation rate of 250 rpm and 39 °C. This temperature was selected based on the results of a study by Jaén et al. (2013). The authors evaluated cell growth and pDNA production at constant temperatures ranging from 30 °C to 45 °C using the strain DH5α. They found that culture at 39 °C allowed an increased pDNA yield on biomass ($Y_{pDNA/OD}$), while only slightly affecting the growth rate compared to culture at 37 °C (Jaén et al. 2013). The composition of the LB was (in g/L): tryptone, 10; yeast extract, 5; NaCl, 10; the composition of the TB was (in g/L): tryptone, 12; yeast extract 24; K_2HPO_4, 9.4; KH_2PO_4, 2.2, plus 4 mL/L of glycerol. Both media were adjusted to pH 7.4 before being sterilized in an autoclave. Cell growth was followed by the measurement of optical density (O.D.) at 600 nm. Glucose was measured using a biochemistry analyzer (YSI 2700 Select; Yellow Springs Instruments Co., Yellow Springs, OH). Samples for the pDNA and acetate analyses of cultures in conventional media were taken at 7 and 10 h of culture for experiments in LB and TB medium, respectively. These time points correspond to the stationary growth phase and represent the harvest times. For cultures in EBP, samples were taken after 18 h of culture, as indicated by the manufacturer. Acetate concentration was determined using the acetic acid UV-method (R-Biopharm, Roche, Darmstadt, Germany) according to the instructions of the manufacturer. pDNA was extracted using a commercial kit based on alkaline lysis and column purification (QIAprep Spin Mimiprep kit; Qiagen, Hilden, Germany) and quantified spectrophotometrically using a NanoDrop 2000c UV-Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The supercoiled fraction was determined by image analysis of pDNA bands separated by agarose gel electrophoresis as described elsewhere (Jaén et al. 2013).

The growth profiles of *E. coli* bearing the different plasmids in LB, TB, and EBP media are shown in Fig. 1a–c, and

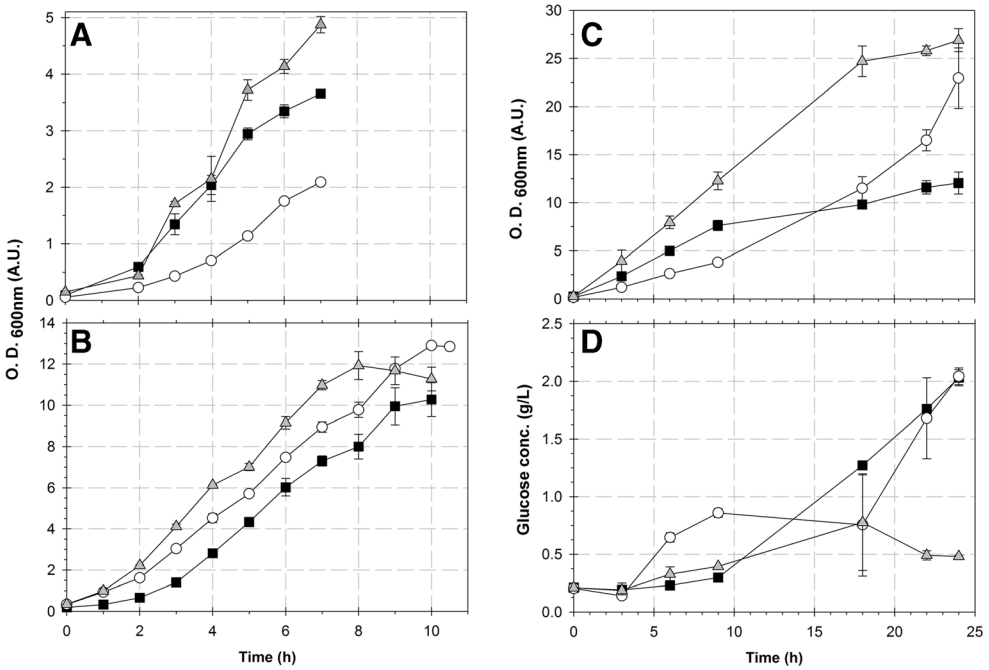

Fig. 1 Growth profiles of the strains bearing the different plasmids. **a**–**c** Growth in Lysogenic Broth (LB) medium (**a**), Terrific Broth (TB) medium (**b**), and EnPresso B Plasmid (EBP) medium (**c**). **d** Glucose accumulation in cultures grown in EBP medium. *Filled squares* 5.4-kbp plasmid, *open circles* 6.0 kbp plasmid, *gray-shaded triangles* 7.8 kbp plasmid. All cultures were carried out in duplicate. *Error bars* show the experimental error between duplicates

glucose accumulation in EBP medium is shown in Fig. 1d. Figure 2a–c shows the specific growth rates in the different media, the acetate accumulation, and the final pH. The parameters related to pDNA production are depicted in Fig. 2d–f.

The most important differences in biomass accumulation between the different plasmids when cultured in conventional media were seen in LB medium, where the maximum accumulation of biomass was observed for the strain bearing the 7.8-kbp plasmid, followed by the strain bearing the 5.4-kbp plasmid and the strain bearing the 6.0-kbp plasmid (Fig. 1a). In LB medium, the strain bearing the smaller plasmid accumulated biomass faster than the other two strains, growing at a rate of nearly 20 % higher, while the growth rate of the strains bearing the 6.0- and 7.8-kbp plasmids, respectively, were nearly equal (Fig. 2a). In LB medium, the growth rates correclated directly with acetate accumulation: whereas the specific growth rates were 0.80 ± 0.00, 0.63 ± 0.02 and 0.67 ± 0.04 h^{-1} for the 5.4-, 6.0-, and 7.8-kbp plasmids, respectively, acetate accumulated up to 0.39 ± 0.04, 0.09 ± 0.01 and 0.19 ± 0.02 g/L in the same order (Fig. 2b). In LB medium, the final pH was lower (5.5 units) in the culture with higher acetate accumulation than in the other two cultures (Fig. 2c).

The amount of pDNA produced in LB medium was inversely proportional to the size of the plasmid: in the culture with the smaller plasmid, 13.2 mg/L of pDNA was produced; in comparison, only 20 and 12 % of this amount of pDNA was produced in cultures with the 6.0- and 7.8-kbp plasmids, respectively (Fig. 2d). This result clearly exemplifies the issue of producing large plasmids at small scale. The negative impact of plasmid size on plasmid content in cells has also been observed by Smith and Bidochka (1988). The $Y_{pDNA/OD}$ was also inversely proportional to plasmid size in LB medium (Fig. 2e). These results indicate that biomass, rather than pDNA synthesis, is favored in LB medium with increasing plasmid size, despite the same replication control circuit in the family of plasmids used here. The supercoiled fraction was smaller than 80 % (Fig. 2f and supplementary material S1), which is the recommended value for therapeutic applications (Mairhofer and Lara 2014).

Terrific broth is another complex medium commonly used for laboratory cultures of *E. coli*. Similar to LB, it contains complex nitrogen and carbon sources, but yeast extract and tryptone concentrations are 20 and 480 % higher in TB than in LB. Moreover, TB also contains a phosphate buffer and glycerol as a defined carbon source. The cell densities reached in TB were higher than 10 O.D. units (Fig. 1b), which is more than twofold higher than those obtained in LB medium. In contrast with the result in LB medium, the maximum cell density in TB was reached by the strain bearing the 6.0-kbp plasmid, whereas the lowest value was obtained for the strain bearing the 5.4-kbp plasmid (Fig. 1b), although the differences were not as great as in the LB medium. In the cultures growin in TB, the growth rate increased with increasing plasmid size (Fig. 2a), with the highest growth rate (0.68 ± 0.05 h^{-1}) observed for the strain bearing the largest plasmid

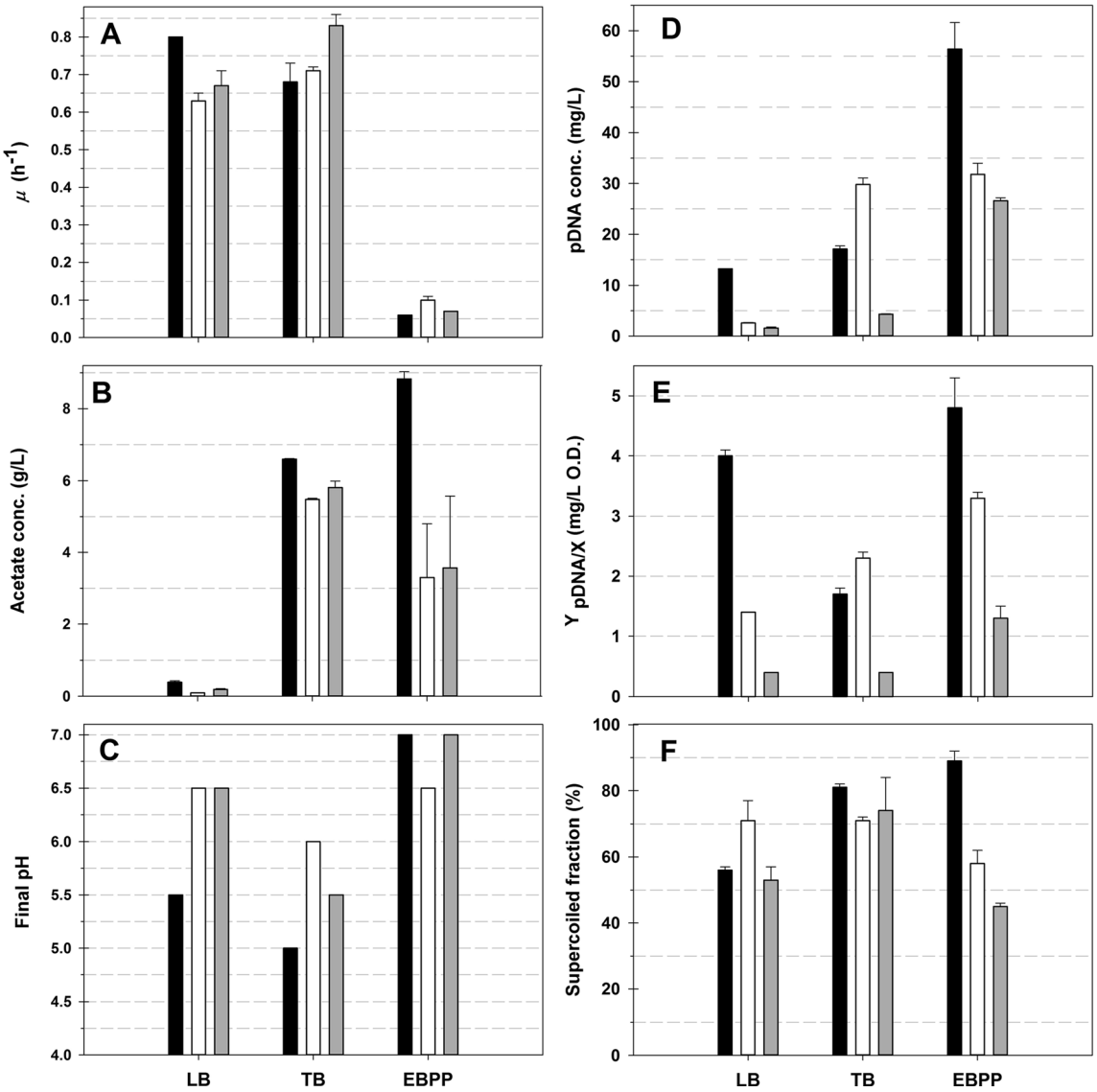

Fig. 2 Main parameters of the cultures and characteristics of plasmid DNA production in the different media. **a** Specific growth rate, **b** acetate concentration in the broth, c final pH of the broth, **d** pDNA concentration, **e** pDNA yield on biomass ($Y_{pDNA/OD}$), **f** supercoiled pDNA fraction. All cultures were carried out in duplicate. *Error bars* show the experimental error between duplicates. *Black bars* 5.4-kbp plasmid, *open bars* 6.0-kbp plasmid, *gray-shaded bars* 7.8-kbp plasmid

(7.8 kbp); in comparison, the strains bearing the 6.0- and 5.4-kbp plasmids had 15 and 18 % lower growth rates, respectively (Fig. 2a). Acetate accumulated to values much higher than those in the LB medium but were similar (nearly 6 g/L) for all three strains (Fig. 2b); this impacted the final pH, which was between 6.0 and 5.0 (Fig. 2c). Neither pDNA production nor the $Y_{pDNA/OD}$ in TB medium showed a clear trend in relation to the plasmid size (Fig. 2d, e). Contrary to cultures in LB, when grown in TB medium, the highest $Y_{pDNA/OD}$ and pDNA titer were observed for the strain bearing the 6.0-kbp plasmid. For all plasmids, pDNA production was higher in TB than in LB medium, and the most important increase in pDNA production was observed for the 6.0-kbp plasmid, which reached a concentration tenfold higher than in TB than in LB (Fig. 2d). Interestingly, the production of the 7.8-kbp plasmid was 2.7-fold higher in TB than in LB medium; nevertheless, the amount of pDNA produced in TB (4.3 ± 0.01 mg/L; Fig. 2d) is still very low for further biotechnology applications. The greater pDNA production in TB, compared to LB, could be explained by the higher availability of asparagine, glutamate, glycine, histidine, leucine (present in the yeast extract), and phosphorus, all of which are needed for DNA synthesis (Danquah and Forde 2007; Mairhofer and Lara 2014). It has also been demonstrated that the availability of aromatic amino acids (tyrosine, phenylalanine and tryptophan) in the culture medium strongly impact pDNA yields (Martins et al. 2015). The supercoiled pDNA fraction improved in cultures on TB compared to those on LB (Fig. 2f). Therefore, it can be

concluded that TB is a better medium than LB for pDNA production in shake flask, mainly due to the fact that more biomass can be produced in TB medium.

pDNA production can be improved by increasing the biomass concentration (as in the case of TB vs. LB media) or by increasing the $Y_{pDNA/OD}$. The former approach would have the consequence of increasing the oxygen demand of the culture and the need for pH control since fermentation or overflow by-products would accumulate (Mairhofer and Lara 2014). The latter approach may be achieved by improving the culture medium and manipulating host physiology. For example, in many cases, low growth rates result in an increase of $Y_{pDNA/OD}$ (Singer et al. 2009; Wunderlich et al. 2014). Low growth rates also reduce the specific oxygen uptake, which in turn can help to increase cell densities. However, the usual strategy to maintain low growth rates is to control the feeding of substrate to the bioreactor, which can be rarely done in shake flasks since it requires additional infrastructure (Weuster-Botz et al. 2001). Slow glucose release to the medium is an option to achieve higher cell densities and pDNA yields, either by uncontrolled release from hydrogels (Sanil et al. 2014) or through an enzyme-controlled mechanism (Grunzel et al. 2014). However, to the best of our knowledge, there are no reports on whether the use of such methods improves the production of large plasmids. Figure 1c shows the growth profiles of the strains bearing the three plasmids, and Fig. 1d shows the glucose concentration in the broth in cultures of *E. coli* on EBP medium. As can be seen in Fig. 1c, the growth of the cells was fast during the first 9 h and decreased strongly after 18 h of culture. The cultures were followed until 24 h, when the stationary phase was observed (Fig. 1c). Between 18 and 24 h of culture, the cells grew very slowly (Fig. 2a), which is expected to be beneficial for pDNA production. Although some glucose accumulated from the beginning of the culture in all cases, the strongest accumulation was seen after 15 h (Fig. 1d), indicating the possibility that other nutrients could be limiting biomass formation. The strain bearing the 5.4-kbp plasmid produced less biomass than the strain bearing the other two plasmids, reaching values that were only slightly higher than those obtained using TB medium (Fig. 1c); the accumulation of glucose was also faster (after 9 h) in the cultures of this strain (Fig. 1d). The cell density of the strain bearing the 6.0-kbp plasmid almost doubled that achieved using TB medium and strong glucose accumulation was also seen after 18 h of culture. The cell density of the culture of the strain bearing the largest plasmid was 2.3-fold higher than that achieved in TB medium (Fig. 1c). This strain grew faster and more stably than the other two plasmids and less glucose was accumulated in the broth (Fig. 1d). Samples for pDNA analyses were taken at 18 (as recommended by the manufacturer), 22, and 24 h of culture in the EBP medium. Higher pDNA concentrations were found at 18 h (Fig. 2d). The 5.4- and 7.8-kbp plasmids produced much higher concentrations of pDNA in the EBP medium than in LB or TB media; in contrast, the amound of pDNA produced by the 6.0-kbp plasmid in EBP medium was almost the same as in TB medium (Fig. 2d). This result is a consequence not only of a higher biomass accumulation, but also of increased $Y_{pDNA/OD}$ values, which were higher in the EBP medium than in LB or TB for all three plasmids (Fig. 2e). The 5.4-kbp plasmid achieved the highest pDNA concentration, which is a concentration well above the reported values obtained using slow glucose release systems (Grunzel et al. 2014; Sanil et al. 2014). Moreover, the pDNA production of the 7.8-kbp plasmid was significantly improved in EBP medium, with the concentrations achieved being 6.2- and 16.6-fold higher than those reached in LB or TB media, respectively. The supercoiled fraction of the obtained pDNA using the EBP medium was higher only in the case of the 5.4-kbp plasmid (Fig. 2f and supplementary material S1). Similar to cultures in LB and TB media, acetate accumulation in EBP medium was higher in the culture of the strain bearing the smaller plasmid, followed by the strain bearing the 6.0-kbp plasmid and the one bearing the largest plasmid (Fig. 2b). When using EBP medium, acetate accumulation reached nearly 9 g/L for the strain bearing the smaller plasmid (Fig. 2b), while for the other two strains acetate concentration was even lower than in TB medium. Despite the relatively high cell densities achieved and the acetate accumulated, the final pH of the EBP medium was nearly neutral (Fig. 2c), which indicates that the medium has a good buffering capacity. The accumulation of acetate when using the EBP medium implies that glucose release rate is faster than the consumption rate. It is possible that other components of the medium could be provisioning the tricarboxylic acid cycle, which, together with high glucose supply, may contribute to acetyl CoA accumulation and therefore to an increase in the overflow metabolism, which results in higher acetate accumulation. Therefore, the formulation of the EBP medium and glucose release rate can be further optimized, taking into consideration the size of the plasmid to produce. Another option to improve pDNA production when using the EBP medium is to combine it with engineered *E. coli* strains with better metabolic performance (Martins et al. 2015; Wunderlich et al. 2014).

Overall, the enzyme controlled glucose release resulted in higher cell densities and higher pDNA yields for all three plasmids, compared to standard culture media. Also, the use of this system resulted in a remarkably improved production of the 7.8-kbp plasmid, thus representing a valuable alternative for the production of large plasmids at small scale in shake flasks. Additionally, our results suggest that reducing the size of the vector could be a viable strategy to improve pDNA production.

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