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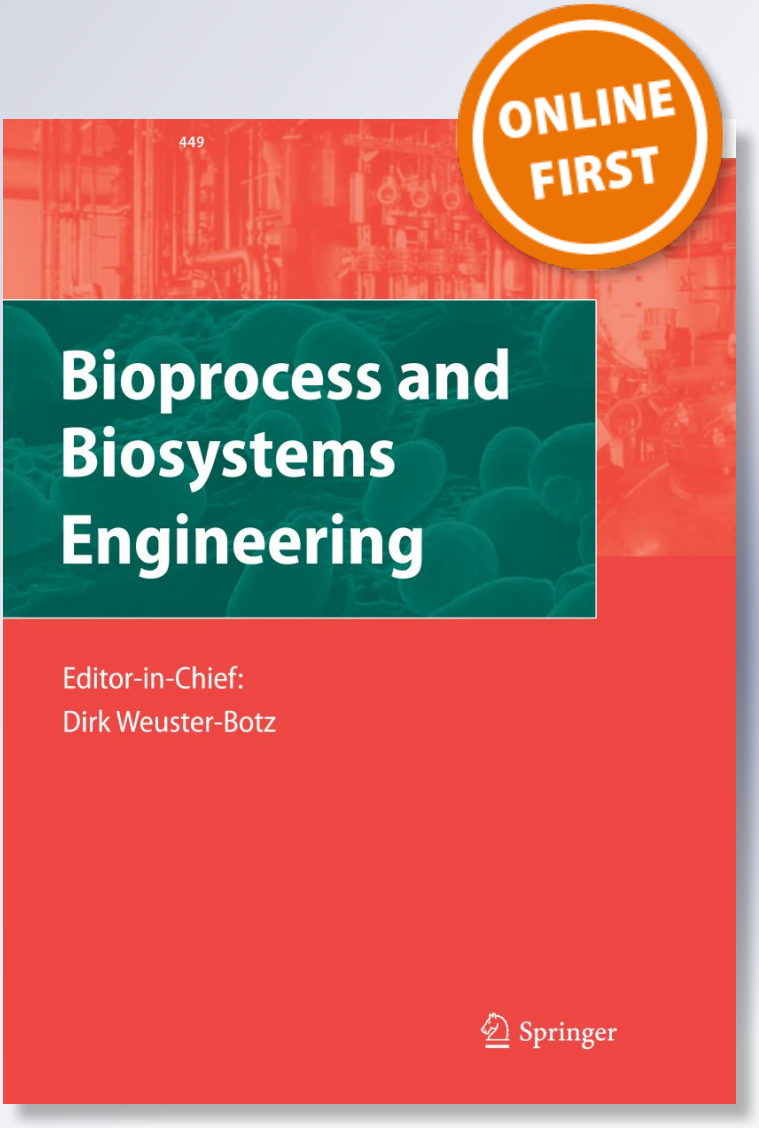

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RESEARCH PAPER

Engineering *E. coli* for improved microaerobic pDNA production

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Abstract
Escherichia coli strains W3110 and BL21 were engineered for the production of plasmid DNA (pDNA) under aerobic and transitions to microaerobic conditions. The gene coding for recombinase A (*recA*) was deleted in both strains. In addition, the *Vitreoscilla* hemoglobin (VHb) gene (*vgb*) was chromosomally inserted and constitutively expressed in each *E. coli recA* mutant and wild type. The *recA* inactivation increased the supercoiled pDNA fraction (SCF) in both strains, while VHb expression improved the pDNA production in W3110, but not in BL21. Therefore, a codon-optimized version of *vgb* was inserted in strain BL21*recA*$^{-}$, which, together with W3110*recA*$^{-}$*vgb*$^{+}$, was tested in cultures with shifts from aerobic to oxygen-limited regimes. VHb expression lowered the accumulation of fermentative by-products in both strains. VHb-expressing cells displayed higher oxidative activity as indicated by the Redox Sensor Green fluorescence, which was more intense in BL21 than in W3110. Furthermore, VHb expression did not change pDNA production in W3110, but decreased it in BL21. These results are useful for understanding the physiological effects of VHb expression in two industrially relevant *E. coli* strains, and for the selection of a host for pDNA production.

Keywords Oxygen limitation · Redox sensor green · Plasmid DNA · *Vitreoscill*a hemoglobin

Introduction

Bioprocess development requires the availability of robust strains that can contend with the environmental conditions that prevail at large scales. The production of commercial molecules often require attaining high cell densities, which results in a high demand of oxygen that can hardly be satisfied due to operational restrictions. Therefore, local or global microaerobic conditions can easily arise [1]. In the case of *Escherichia coli* cultures, exposure to microaerobic conditions triggers the synthesis of acidic by-products, decreasing the capacity for biomass and product formation. However, microaerobic conditions can increase pDNA yields [2, 3], which may be useful provided that proper cell factories are available. There have been several efforts to improve the performance of *E. coli* under microaerobic conditions, for instance, inactivating fermentative pathways [4] or expressing a heterologous hemoglobin that improves the metabolism under oxygen limitation [5–8]. The applications of such strategies have not been thoroughly tested for the production of plasmid DNA (pDNA), which is a molecule of potential application as a therapeutic agent [9].

pDNA production at large-scale is usually carried out in high cell-density cultures using specialized strains and culture media [10–12]. High cell densities are normally attained using fed-batch schemes. Fed-batch strategies using enzyme-controlled glucose release have been applied in small-scale cultures to enhance pDNA production, which has proved to be attractive for laboratory applications [13–15]. While oxygen limitation can also occur in such small-scale systems [14, 15], the use of oxygen vectors is useful to overcome this limitation [16]. However, the feasibility of such strategy for large-scale pDNA production has not been proved. It has been reported that the use of engineered cells with reduced overflow metabolism can reach high cell densities and attractive pDNA yields in batch mode using high initial glucose concentrations [17, 18]. However, oxygen limitation

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can be stronger in such cultures than in fed-batch mode, due to the presence of glucose excess during most of the culture process. The presence of VHb radically improves the culture performance under such conditions [7, 8]. Nevertheless, the effect of VHb on pDNA production under oxygen limitation in high cell-density cultures in batch mode has not been reported.

The common hosts used for pDNA production include specialized strains such as DH5α and DH10B [12, 19]. Those strains contain a dozen mutations to enhance pDNA yield and stability and to decrease its degradation. Such a high number of mutations may cause unpredictable effects under large-scale conditions. Compared to wild-type strains, the physiology of strain DH5α has been poorly characterized. For instance, it was demonstrated that DH5α is not a *deoR* mutant (*deoR* is a gene related to the nucleotide synthesis), as previously stated [20]. Moreover, it has been shown that the pDNA production as well as the supercoiled pDNA fraction (SCF) strongly varies upon the *E. coli* strain used [21, 22], which more probably depends on the metabolic and regulatory structure [23]. The SCF is a relevant factor to assess the quality of the pDNA produced, since high content of supercoiled pDNA is preferred for eliciting therapeutic functions [9]. Hence, the application of metabolic engineering strategies in different genetic backgrounds can lead to different results. From the diversity of *E. coli* strains, W3110 and BL21 (DE3) are two of the most widely used for research and industrial purposes and have been extensively studied [23–26]. An interesting characteristic of strain BL21 is that it displays a low acetate synthesis under aerobic conditions, which has been attributed to particular features of the tricarboxylic acid cycle (TCA) function in this strain [25]. However, the performance of this strain for pDNA production has been only scarcely studied. In the present study, we analyze the effect of deleting the *recA* gene in *E. coli* strain W3110 and BL21. Recombinase A plays several functions on DNA repair and SOS response [27]. The positive effects of *recA* inactivation on the production of pDNA have been demonstrated in strains such as MG1655 [22], BL21 [28], and W3110-derived strains [18]. The inactivation of *recA* has been reported to reduce the amount of topoisomers and multimers, thus resulting in a more homogeneous plasmid. In addition, the *Vitreoscilla* hemoglobin (VHb) gene was inserted in the chromosome of wild-type and *recA* mutant strains. It has been widely documented that VHb improves microaerobic growth and metabolism of a variety of microorganisms [5]. In this work, cell growth, pDNA yields and topology, and extracellular metabolite analyses of engineered *E. coli* strains were assessed. Furthermore, the reductase activity and cell viability were monitored in culture with shift from aerobic to oxygen-limited regimes. As an indicator of terminal reductases activity, the Redox Sensor Green (RSG) dye was used. RSG is reduced by the intracellular reductases of the aerobic metabolism [29], releasing a green fluorescent compound that can be easily detected by flow cytometry [30]. Cell viability was monitored using the propidium iodide (PI) staining method [31]. The combination of those techniques allowed to critically evaluate engineered strains and gain knowledge toward the design of better cell factories for pDNA production under microaerobic conditions.

Materials and methods

Strains construction

The wild-type *Escherichia coli* strains used in this study were W3110 and BL21 (DE3). The *recA* gene was inactivated in both strains using the methodology proposed by Datsenko and Wanner [32]. The gene coding for the *Vitreoscillastercoraria* hemoglobin (GenBank: L21670.1) was inserted in the chromosome of each strain according to the methodology developed by Sabido et al. [33]. This methodology uses the pLoxGentrc plasmid, in which the *vgb* gene was located downstream the *trc* promoter (P_{trc}). This way, the *vgb* gene under the P_{trc} control was integrated into the chromosome by homologous recombination between *lacI* and *lacZ*. The integration was confirmed by resistance to gentamicin, the absence of blue coloration in colonies grown in X-Gal plus 1 mM IPTG LB plates and PCR tests. The interruption of *lacI* and *lacZ* and the introduction of the P_{trc} yield the expression of *vgb* constitutive. The modified version of the vgb gene (GenBank: L21670.1) was obtained by codon optimization for *E. coli* using the Optimum Gene codon optimization tool by GenScript (Piscataway, NJ, USA). The optimized gene was inserted into the chromosome of *E. coli* BL21 as described above. Both versions of the *vgb* gene were synthesized by GenScript (Piscataway, NJ, USA). All the constructions were probed by proper PCR analyses. All the strains were transformed with the pVAX1 plasmid (Invitrogen, Carlsbad, CA, USA) to test pDNA production, which contains a kanamycin resistance gene.

Culture medium and precultures' development

For precultures development, cryo-preserved cells were inoculated in 250 or 500 mL shake flasks containing mineral medium with the following composition (in g/L): K_2HPO_4, 17; KH_2PO_4, 5.3; $(NH_4)_2SO_4$, 2.5; NH_4Cl, 1.0; citrate–$Na_3{\cdot}2H_2O$, 2; $MgSO_4{\cdot}7H_2O$, 1.0; thiamine–HCl, 0.01. The medium was supplemented with and trace-element solution, 2 mL/L and 50 µg/mL kanamycin sulfate. The trace-element solution composition (in g/L) was $ZnCl_2$, 10.5; EDTA, 5.5; $CoSO_4{\cdot}7H_2O$, 1.5; $MnSO_4{\cdot}H_2O$, 6.4; $CuSO_4{\cdot}5H_2O$, 1.1; H_3BO_3, 1.5; $Na_2MoO_4{\cdot}2H_2O$, 1;

$FeCl_3 \cdot 6H_2O$, 51.4; and Cit-H·H_2O, 39.9. Glucose was added at a final concentration of 5 (for 250 mL shake flasks) or 6.25 (for 500 mL shake flasks) g/L. The latter was used to reach higher biomass concentrations for inoculation of the main culture. Kanamycin sulfate was used at 50 mg/L. The shake flasks were incubated during 12–16 h, in orbital shakers at 250 (for 250 mL shake flasks) or 170 (for 500 mL shake flasks) rpm, with an orbital diameter of 50 mm. After the incubation period, the broth was collected and centrifuged at 7500 rpm for 10 min and the cell pellet was washed and resuspended in 3 mL of fresh medium. These washed cells were used to inoculate the bioreactors for the main cultures. The bioreactor cultures contained the medium described above, supplemented with 5 or 10 g/L of glucose, as stated in each case.

Cultures in shake flasks

Shake flasks of 250 mL volume with 4 baffles in the bottom were used. The shake flasks were filled with 50 mL of medium plus 5 g/L of glucose, inoculated at an initial optical density (600 nm) of ca. 0.2 units, and capped with sponge closures. The cultures were performed in an orbital shaker at 37 °C, 300 rpm and shaking diameter of 50 mm. Cell growth was followed as absorbance at 600 nm using a BioPhotometer Plus (Eppendorf, Wesseling-Berzdorf, Germany).

Bioreactor cultures

Cultures with transition from aerobic to oxygen-limited conditions were performed in a 4 stirred-tank minibioreactors platform (DASGIP DASbox Reactor SR0250ODLS, Eppendorf AG, Hamburg, Germany). Each bioreactor contained 200 mL of medium supplemented with 12 g/L of glucose and operated at 37 °C and pH 7.2 (controlled by the addition of NH_4OH or H_3PO_4) and air-flow rate of 0.85 vvm. DOT was measured using PSt1 optical sensors linked to an OXY-4 oxygen meter (Presens Precision Sensing, Regensburg, Germany). Stirring rate was set at 1250 rpm during the first 4 h of culture to ensure full aerobic regime and then decreased to 750 rpm to induce oxygen limitation.

Offline analyses

Biomass and metabolite concentrations

Cell growth in stirred bioreactors was measured optical density as described above. When expressing parameters in terms of biomass concentration, the optical density was multiplied by a pre-determined factor to convert to g/L dry cell weight. Glucose concentration was measured in an YSI 2700 biochemistry analyzer (YSI Inc., OH, USA). Organic acids were analyzed by HPLC using a Bio-Rad Aminex HPX-87H column (Bio-Rad Laboratories Inc., CA, USA) at 50 °C and 0.4 mL/min of 5 mM H_2SO_4, and a UV detector set at 210 nm.

pDNA analyses

pDNA was isolated and purified from 5.8 mg of wet biomass using the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany), and recovered in 70 µL of EB buffer at 70 °C. Such procedure enabled to maximize the amount of pDNA extracted from cells (data not shown), whereas the supercoiled fraction (SCF) is not expected to be influenced, as indicated by the manufacturer. The extracted pDNA was quantified in a Nanodrop 2000 (Thermo Fisher Scientific, WI, USA) system. The SCF was determined from the image analysis of 0.8% agarose gels pre-stained with SYBR green safe (Invitrogen, Carlsbad, USA).

Flow cytometry, PI, and RSG staining

Cell viability was measured by flow cytometry of cells samples stained with PI (Thermo Fisher Scientific, WI, USA). RSG (Thermo Fisher Scientific, WI, USA) was used as indicator of reductases activity. The fluorescence of stained cells was measured in C6 Accuri Flow Cytometer (BD Biosciences, NJ, USA). Details on the staining procedure and mathematical treatment of data can be found in a previous report [30].

Results and discussion

pDNA production aerobic cultures of *recA*$^-$ strains and *recA*$^-$*vgb*$^+$ strains

The effect of *recA* deletion was first tested in *E. coli* W3110 and BL21. Figure 1 shows the main parameters calculated during the exponential growth phase. Wild-type strains are denoted as *recA*$^+$, while mutant strains as *recA*$^-$. In W3110, the specific growth rate (µ) and biomass yield on glucose ($Y_{X/S}$) seemed unaffected by the *recA* deletion (Fig. 1a). Nevertheless, the plasmid yield on biomass ($Y_{pDNA/X}$) fell from 1.60 ± 0.20 in wild-type W3110 to 0.90 ± 0.09 mg/g in W3110*recA*$^-$ (Fig. 1c). These results differ from those shown in a previous report using a substrate transport mutant of strain W3110, in which the inactivation of *recA* increased the $Y_{pDNA/X}$ more than twofold. Moreover, the SCF in the aforementioned report was always lower than 70% [18]. However, the strains used in the previous report carried modifications (PTS$^-$GalP$^+$) affecting significantly substrate transport, making difficult a direct comparison of the results. In contrast, the SCF was 40% in W3110*recA*$^+$ and increased up to 83% when *recA* was inactivated (Fig. 1e). These results

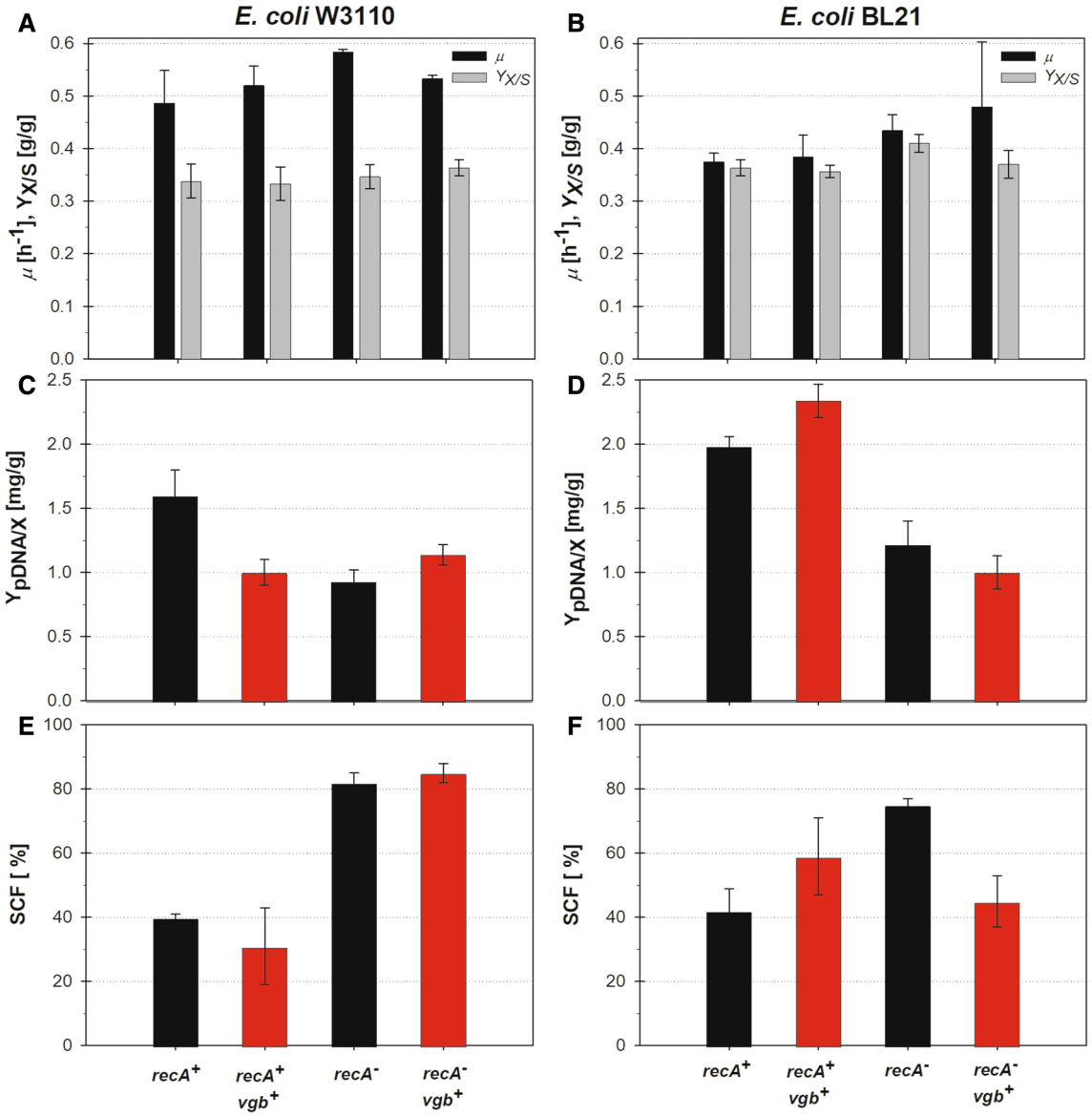

Fig. 1 Aerobic growth and pDNA production characteristics of the wild type and engineered *E. coli* strains. Cultures were performed in mineral medium supplemented with 5 g/L of glucose using baffled shake flasks. **a**, **b** Specific growth rate and biomass yield on glucose; **c**, **d** pDNA yields on biomass; **e**, **f** supercoiled pDNA fraction (SCF). Samples for pDNA analysis were taken during the exponential growth phase. Error bars show one standard deviation of triplicate cultures

are in agreement with the expected effects of deleting *recA*, as explained below. The effect of expressing VHb was tested separately in the wild type and *recA* mutant. The presence of VHb in W3110 had little effect on μ and $Y_{X/S}$ of the wild-type of *recA* mutant (Fig. 1a). The $Y_{pDNA/X}$ decreased from 1.60 ± 0.20 in W3110*recA*$^+$ to 1.00 ± 0.10 mg/g in W3110*recA*$^+$*vgb*$^+$ (Fig. 1c), while increased *ca.* 23% in W3110*recA*$^-$ compared with W3110*recA*$^-$ (Fig. 1c). These results differ from those from Pablos and coworkers [7] that reported a twofold $Y_{pDNA/X}$ increase in W3110 expressing the VHb compared with its wild type. A possible reason for such discrepancy is that the former report used a medium copy-number phagemid to express *vgb*. It is expected to have one *vgb* gene of the engineered strain as a result of the single insertion in the chromosome. In contrast, for the phagemid pBS (ColE1 origin of replication), the copies of vgb per cell can vary between 300 and 500. Therefore, it can expected a larger amount of active VHb in the plasmid-based system, by comparison with the chromosomal insertion reported here.

The behavior of the BL21 strain was different. $Y_{X/S}$ were slightly higher for BL21*recA*$^+$, compared with W3110*recA*$^+$, and the *recA* inactivation had little effect on this yield (Fig. 1b). In general, the wild-type and mutant versions of BL21 displayed a lower μ than W3110 (Fig. 1a,

b). Deletion of *recA* had only slight effects on μ and $Y_{X/S}$ of BL21 (Fig. 1b). In contrast, $Y_{pDNA/X}$ decreased by 38% (from 1.98 ± 0.08 to 1.22 ± 0.18 mg/g) due to *recA* deletion in BL21 (Fig. 1d). Although a diminution of $Y_{pDNA/X}$ as a result of *recA* inactivation was also found for W3110 (Fig. 1c), such effect differ with data from a previous report. Phue and Shiloach [28] also inactivated *recA* in strain BL21, but $Y_{pDNA/X}$ of a high copy-number plasmid did not notoriously changed when the culture was maintained at 30 °C. This may be related to the culture medium, since the authors used a semi-defined medium with tryptone and yeast extract, which can mask some physiological effects of gene inactivation. Similar to the results of W3110, the deletion of *recA* had a strong positive effect on plasmid homogeneity. The wild-type BL21*recA*$^+$ produced plasmid with a SCF of 42 ± 7%, while the mutant BL21*recA*$^-$ increased the quality up to a SCF of 75 ± 2% (Fig. 1f). Yet, this value is not high enough to comply with the FDA recommendations [34]. Expression of VHb had only minor effects on μ and $Y_{X/S}$ of BL21 (Fig. 1b). However, the mutant BL21*recA*$^+$*vgb*$^+$ reached an $Y_{pDNA/X}$ of 2.34 ± 0.13 mg/g, which was 18% higher than that of the wild-type BL21*recA*$^+$ and the highest of all the studied strains (Fig. 1c, d). Nonetheless, the combination of *recA* inactivation and *vgb* expression resulted in an $Y_{pDNA/X}$ of 1.00 ± 0.13 mg/g that is the lowest of all the BL21 strains studied (Fig. 1d). Furthermore, the strain BL21*recA*$^-$*vgb*$^+$ produced the most heterogeneous pDNA, with a SCF of only 45 ± 8% (Fig. 1f).

In general, the inactivation of *recA* positively impacted the topology of the pDNA produced in BL21 and W3110 strains. This effect can be indirect by a combined action of topoisomerase I and gyrase A. The former relax the DNA, while the latter introduces negative supercoiling in the DNA, consuming ATP [35]. It has been shown that RecA stimulates the activity of topoisomerase A in vivo [36]; therefore, deletion of *recA* may decrease the activity of topoisomerase A, thus producing more negatively supercoiled pDNA. Gene expression is less efficient if the DNA supercoiling increases [36, 37], since access to the promoter region and polymerase displacement is hindered. Therefore, it is possible that the higher SFC of the pDNA produced in *recA* mutants could difficult the expression of the positive replication control molecule, rnaII. This may in part explain the lowered $Y_{pDNA/X}$ in *recA* mutants. Notwithstanding this decrease of $Y_{pDNA/X}$, the *recA* mutation is to be conserved in strains for pDNA production.

Strain BL21 seemed a better producer than W3110. However, the pDNA quality (measured as SCF) is too low. The effect of VHb expression under aerobic conditions was clearer in BL21, still, the SCF of the double mutant is far from the recommended value of 80% [34]. As a possibility to enhance the effect of VHb from the transcriptional level, the *vgb* gene was codon-optimized. Figure 2 shows a comparison of the wild-type and optimized *vgb* sequences. The optimized sequence, denoted as *, was then inserted in the chromosome of strain BL21*recA*$^-$ to obtain the strain BL21*recA*$^-$*vgb**. This strain was directly evaluated in cultures with transitions to microaerobic conditions as described below.

pDNA production in cultures with shifts from aerobic to oxygen-limited regimes cultures of W3110-derived strains

The strains W3110*recA*$^-$, W3110 *recA*$^-$*vgb*$^+$, BL21*recA*$^-$, and BL21*recA*$^-$*vgb** were evaluated for pDNA production in cultures with transitions from aerobic to microaerobic conditions. To gain further insight in the physiological responses, the activity of terminal oxide reductases and cell viability was monitored by flow cytometry. The kinetic profiles of W3110-derived strains are shown in Fig. 3. The vertical dotted line shows the point of stirring rate reduction, which resulted in the decrease of DOT from around 30% to *ca*. 0% air sat. (Fig. 3f). During the aerobic phase of the cultures (DOT $\geq$ 20% air sat.), the growth rates were similar for the strains expressing (0.45 ± 0.02 h^{-1}) or non-expressing (0.39 ± 0.07 h^{-1}) VHb. The optical density readings showed strong variations by the end of the culture. This may be caused by changes in cell shape that and size due to microaerobiosis that could affect the

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Optimized 1-ATGCTGGACCAACAGACCATCAACATCATCAAAGCGACGGTTCCGGTGCTGAAAGAACAC
Original  1-ATGTTAGACCAGCAAACCATTAACATCATCAAAGCCACTGTTCCTGTATTGAAGGAGCAT
Optimized   GGCGTTACCATTACCACGACGTTTTACAAAAACCTGTTTGCCAAACATCCGGAAGTTCGT
Original    GGCGTTACCATTACCACGACTTTTTATAAAAACTTGTTTGCCAAACACCCTGAAGTACGT
Optimized   CCGCTGTTCGATATGGGCCGCCAGGAAAGCCTGGAACAACCGAAAGCACTGGCAATGACC
Original    CCTTTGTTTGATATGGGTCGCCAAGAATCTTTGGAGCAGCCTAAGGCTTTGGCGATGACG
Optimized   GTCCTGGCAGCAGCACAGAACATTGAAAATCTGCCGGCAATCCTGCCGGCTGTGAAGAAA
Original    GTATTGGCGGCAGCGCAAAACATTGAAAATTTGCCAGCTATTTTGCCTGCGGTCAAAAAA
Optimized   ATTGCAGTTAAACATTGCCAGGCTGGTGTCGCTGCAGCACACTATCCGATTGTGGGCCAA
Original    ATTGCAGTCAAACATTGTCAAGCAGGCGTGGCAGCAGCGCATTATCCGATTGTCGGTCAA
Optimized   GAACTGCTGGGTGCGATCAAAGAAGTGCTGGGTGATGCAGCTACGGATGACATCCTGGAC
Original    GAATTGTTGGGTGCGATTAAAGAAGTATTGGGCGATGCCGCAACCGATGACATTTTGGAC
Optimized   GCGTGGGGCAAAGCCTACGGTGTTATCGCAGACGTGTTTATTCAGGTGGAAGCAGACCTG
Original    GCGTGGGGCAAGGCTTATGGCGTGATTGCAGATGTGTTTATTCAAGTGGAAGCAGATTTG
Optimized   TACGCACAGGCAGTGGAATGA-441
Original    TACGCTCAAGCGGTTGAATAA-441
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Fig. 2 Comparison of the native and codon-optimized sequence for *E. coli* of the *Vitreoscilla* hemoglobin. The changes in the optimized sequence are shown in red (color figure online)

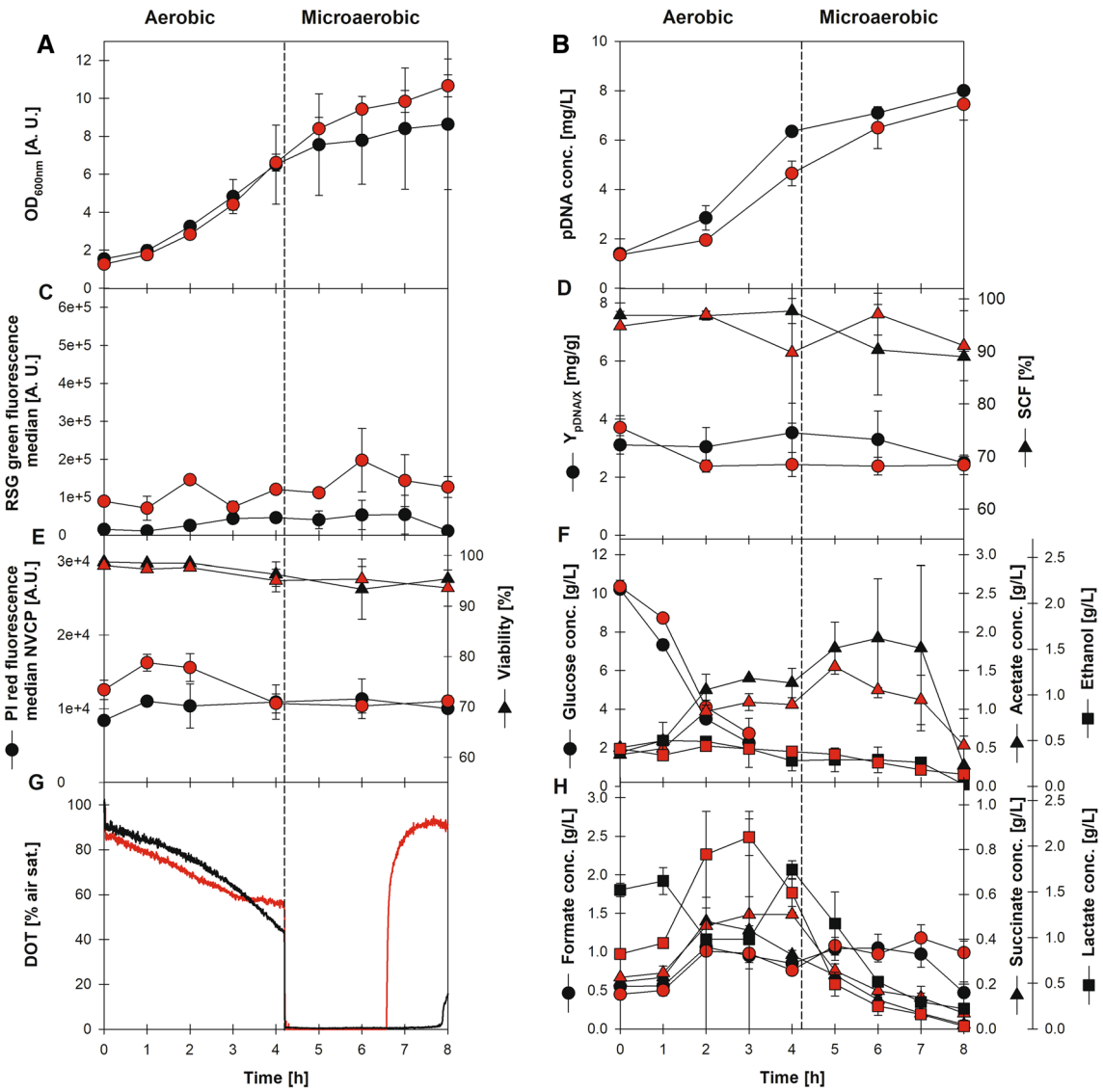

Fig. 3 Growth profiles of biphasic of *E. coli* strains W3110 *recA*$^-$ (black symbols) and W3110 *recA*$^-$*vgb*$^+$ (red symbols) cultured in minibioreactors. **a** Cell growth; **b** pDNA concentration; **c** RSG green fluorescence median; **d** pDNA yield on biomass and supercoiled fraction; **e** PI red fluorescence signal median and cell viability; **f** glucose, acetate, and ethanol concentration; **g** DOT (black line: W3110 *recA*$^-$; red line: W3110 *recA*$^-$*vgb*$^+$); **h** formate, succinate, and lactate concentration. Vertical dotted lines indicate the time of reduction of the stirring rate from 1250 to 750 rpm. Error bars show the experimental error of duplicate experiments (color figure online)

absorbance of the broth. When microaerobic conditions prevailed, the growth of both strains slowed down. However, while W3110*recA*$^-$ almost stopped growing (Fig. 3a), strain W3110 *recA*$^-$*vgb*$^+$ kept growing at a rate of $0.18 \pm 0.04\ h^{-1}$, and reached an optical density 25% higher than that of W3110*recA*$^-$ (Fig. 1a). The redox activity (as monitored by median of the RSG green fluorescence) was three-fold higher for the strain expressing VHb than for the non-expressing strain (Fig. 3c). This is in agreement with the proposed mechanism of action of the VHb, which possibly intensify the activity of the electron transport chain and functions as a terminal oxidase [5]. Interestingly, the median of the RSG green fluorescence remained relatively constant for both strains, regardless the availability of oxygen. The cellular viability, remained higher than 90% during the aerobic and microaerobic phases of the culture for both strains (Fig. 3e). Notwithstanding, the median of the red fluorescence (an indicator of cell damage) was higher during the aerobic phase of cultures of W3110*recA*$^-$*vgb*$^+$, compared with W3110*recA*$^-$, and then was nearly the same for both strains during the microaerobic phase (Fig. 3e). This suggest that strain W3110*recA*$^-$*vgb*$^+$ is more prone to cell damage when the DOT is above 60% from saturation than its parent strain.

During the aerobic phase of the culture, the pDNA production was higher for strain W3110*recA*$^-$ than for W3110*recA*$^-$*vgb*$^+$, but under microaerobic conditions, the VHb-expressing strain accumulated pDNA faster than its parent, and the pDNA concentration was similar for both strains at the end of the culture (Fig. 3b). This occurred due to the higher biomass accumulation of strain W3110*recA*$^-$*vgb*$^+$, compared with W3110*recA*$^-$, since $Y_{pDNA/X}$ was lower for the former than for the latter strain during most of the culture (Fig. 3d). Interestingly, $Y_{pDNA/X}$ of strain W3110*recA*$^-$ decreased upon transition to microaerobic conditions, which differs from the previous reports [2, 3]. In contrast, $Y_{pDNA/X}$ of strain W3110*recA*$^-$*vgb*$^+$ remained relatively constant during the culture (Fig. 3d). The SCF remained close to 90% for both strains, regardless the oxygen availability (Fig. 3d).

The production of metabolic by-products is shown in Fig. 3f, h. Acetate was the major by-product for both strains, during the aerobic and microaerobic phases. The VHb-expressing strains produced less acetate than its parent strain, either by overflow or fermentative metabolism (Fig. 3f), which is consistent with the previous studies [6–8]. Interestingly, the VHb-expressing strain produced relatively high amounts of lactate under aerobic conditions, while the production of formate, succinate (Fig. 3h), and ethanol (Fig. 3f) was similar for both strains. The amount of produced by-products by each strains during the cultures is compared in Fig. 5. It can be seen that the fermentation profiles changed due to VHb expression basically by decreasing the synthesis of acetate and increasing the synthesis of lactate. The reason for this is unclear and deserves further investigation. For instance, Tsai et al. [38] reported an increase on the flux to lactate in *E. coli* W3110 containing VHb at a low concentration (0.5 µmol/g biomass), compared to the wild-type strain. However, when the amount of intracellular VHb increased, the flux to lactate decreased [38]. Detailed metabolic flux analyses of pDNA production in strains expressing VHb could help to clarify the differences in the fermentation profiles.

Taken together, the cultures of W3110-derived strains show that the expression of VHb improves growth rate and redox activity, especially under the microaerobic phase. $Y_{pDNA/X}$ was not changed by the microaerobic conditions, and the final pDNA concentration was very similar compared with the parent strain (7.45 ± 0.64 and 8.0 ± 0.00 mg/L, respectively). Therefore, strain W3110*recA*$^-$*vgb*$^+$ may be a good candidate for pDNA production, particularly if higher cell densities are sought and oxygen depletion is expected.

BL21-derived strains

The growth profiles of strains derived from BL21 are shown in Fig. 4. The growth of the VHB-expressing strain was slower than that of the parent strain (Fig. 4a). This was reflected in a slower consumption of oxygen (Fig. 4g). In fact, due to the fast growth rate of strain BL21*recA*$^-$, dissolved oxygen was depleted 1 h before the programmed reduction of the stirring rate in the cultures of this strain (Fig. 4g). The maximum optical density in cultures of the VHb-expressing strain was 10% lower than the corresponding value for BL21*recA*$^-$ (Fig. 4a). The reductases activity, as reflected by the median of the RSG green fluorescence, was much higher for the VHb-expressing than for the parent strain (Fig. 4c). In strain BL21*recA*$^-$, the RSG green fluorescence decreased through the culture and fell down to a minimum during the microaerobic phase (Fig. 4c). In contrast, it increased during the beginning of the microaerobic phase of strain BL21*recA*$^-$*vgb**, and was up to 30-fold higher than that of BL21*recA*$^-$ (Fig. 4c) and considerably higher than that of W3110*recA*$^-$*vgb*$^+$ (Figs. 3c, 4c). This agrees with a higher respiratory activity deduced by the faster depletion of dissolved oxygen by strain BL21*recA*$^-$*vgb**, compared with the three other strains. The viability of BL21-derived strains was similar and relatively constant during the aerobic phase of the culture (Fig. 4e). Nonetheless, it slightly decreased for strain BL21*recA*$^-$ during the microaerobic phase, while remained constant for BL21*recA*$^-$*vgb** (Fig. 4e). Moreover, the median of the red PI fluorescence was constant for BL21*recA*$^-$*vgb**, and lower than BL21*recA*$^-$ through the culture, which indicates that the VHb contributed to maintain the integrity of the cells.

The microaerobic conditions strongly impacted the production of pDNA in strain BL21*recA*$^-$, as can be seen in Fig. 4b. The pDNA concentration steadily increased during the aerobic phase, but decreased during the microaerobic phase. Despite the higher metabolic activity of strain BL21*recA*$^-$*vgb**, the pDNA production was lower than that of the parent strain. Although pDNA synthesis in strain BL21*recA*$^-$*vgb** seemed not affected by microaerobic conditions, the final pDNA titer was 37% lower (4.80 ± 0.70 mg/L) than the reached by BL21*recA*$^-$ (7.60 ± 0.28 mg/L) (Fig. 4b). $Y_{pDNA/X}$ decreased during transition to microaerobiosis for BL21*recA*$^-$, but remained constant for BL21*recA*$^-$*vgb** (Fig. 4d). The pDNA SCF was higher than 80% in cultures of BL21*recA*$^-$ strain, while reached up to 92% in cultures of the VHb-expressing strain (Fig. 4d). This is a substantial improvement compared with the strain expressing the non-optimized version of the *vgb* gene (Fig. 1f).

As expected, the production of acetate by BL21-derived strains was lower (Fig. 4f). Ethanol was detected during the microaerobic phase for both strains (Fig. 4f). Lactate was also produced during the aerobic phase by both strains (Fig. 4h). Lactate production in recombinant BL21 has also been reported before [39]. Succinate and formate accumulated to minor amounts (Fig. 4h). In general, the accumulation of by-products by BL21-derived was lower than for

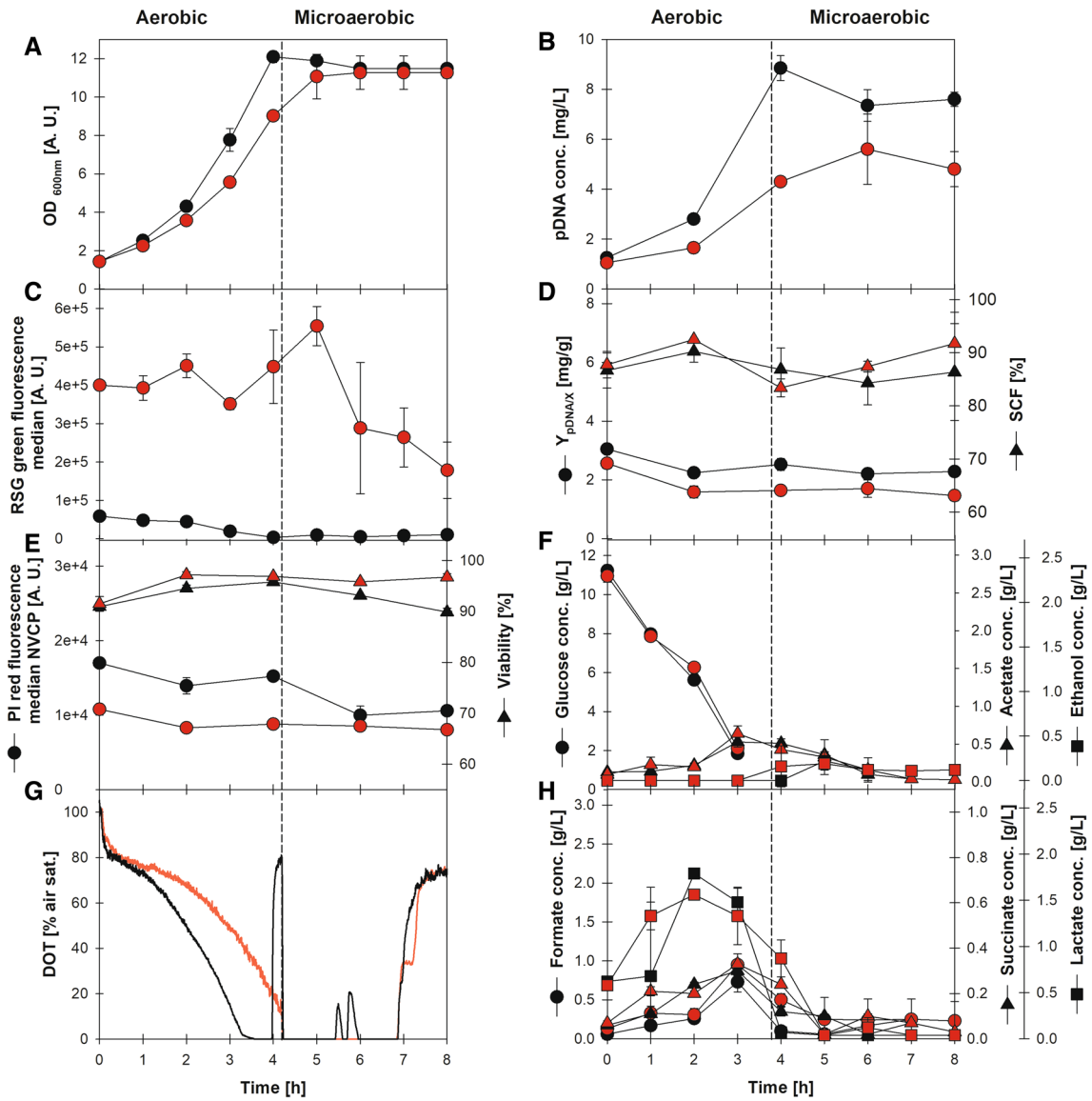

Fig. 4 Growth profiles of biphasic of *E. coli* strains BL21 *recA*$^-$ (black symbols) and BL21 *recA*$^-$*vgb*$^+$ (red symbols) cultured in minibioreactors. **a** cell growth; **b** pDNA concentration; **c** RSG green fluorescence median; **d** pDNA yield on biomass and supercoiled fraction; **e** PI red fluorescence signal median and cell viability; **f** glucose, acetate, and ethanol concentration; **g** DOT (black line: BL21 *recA*$^-$; red line: WBL21 *recA*$^-$*vgb*$^+$); **h** formate, succinate, and lactate concentration. Vertical dotted lines indicate the time of reduction of the stirring rate from 1250 to 750 rpm. Error bars show the experimental error of duplicate experiments (color figure online)

W3110-derived strains (Fig. 5). Contrary to the effect in W3110*recA*$^-$, the expression of VHb did not change the fermentation profile in BL21*recA*$^-$ (Fig. 5). Lactate was the main by-product in cultures of BL21-derived strains, although the expression of the VHb reduced the accumulation of this acid.

The result of expressing the VHb in BL21*recA*$^-$ indicates a higher metabolic activity, together with a decreased accumulation of fermentative by-products. However, this was not accompanied by higher biomass or pDNA production. Nonetheless, the presence of VHb favored the supercoiling of the produced plasmid. Additional genetic modifications to increase pDNA synthesis could be applied to improve these results. For instance, deletion of the *relA* gene, involved in the stringent response to amino acid depletion, can increase the pDNA yields up to tenfold after arginine exhaustion [40, 41]. Therefore, studies of additional mutations and VHb expression in glucose-limited fed-batch cultures under oxygen limitations will help to explore the further evaluate the utility of the proposed approach.

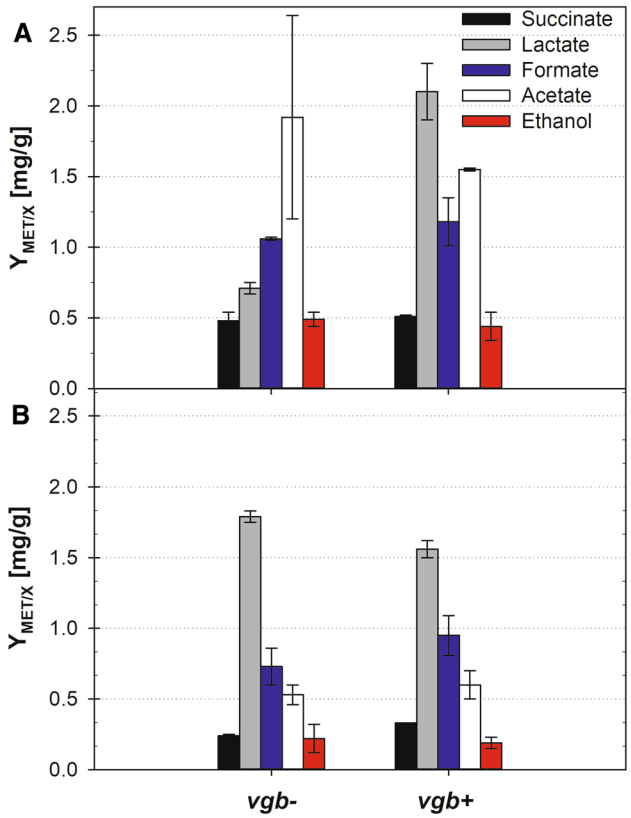

Fig. 5 Maximum concentration of fermentative by-products in cultures of W3110 (**a**) and BL21 (**b**) derived strains expressing (*vgb*$^-$) or non-expressing (*vgb*$^+$) the VHb. Error bars show the experimental error of duplicate experiments

Conclusions

Overall, the results shown here demonstrate that is feasible to improve the behavior of *E. coli* strains for the production of pDNA when oxygen depletion arises in a bioreactor. The inactivation of *recA* efficiently increased the pDNA SCF in both strains. The physiological responses induced by VHb expression, although beneficial, were not equivalent in BL21 and W3110. Despite BL21*recA*$^-$*vgb** accumulated less organic acids than W3310*recA*$^-$*vgb*$^+$, the latter strains performed better for pDNA production. Therefore, such strain is proposed as a candidate for further development of pDNA production processes under microaerobic conditions, like those that frequently arise in large-scale bioreactors.

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Compliance with ethical standards

Conflict of interest The authors declare that they have noconflict of interest.

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