Effect of the oxygen transfer rate on oxygen-limited production of plasmid DNA by *Escherichia coli*

Alvaro R. Lara[1*], Karim E. Jaén[1], Olusegun Folarin[2], Eli Keshavarz-Moore[2], Jochen Büchs[3]

[1]Departamento de Procesos y Tecnología, Universidad Autónoma Metropolitana-Cuajimalpa, México

[2]Department of Biochemical Engineering, University College London, UK

[3]RWTH Aachen University, AVT-Biochemical Engineering, Germany

*Corresponding author.

Email address: alara@correo.cua.uam.mx

ORCID: 0000-0003-3535-7619

Abstract

The influence of oxygen limitation on pDNA yields in cultures of *Escherichia coli* was studied. Cultures at maximum oxygen transfer rate (OTR_{max}) values of 10, 14, 30 and 45 mmol L^{-1} h^{-1} and an aerobic culture at an OTR_{max} of 110 mmol L^{-1} h^{-1} were performed in microtiter plates (MTPs). Dissolved oxygen tension (DOT), pH, biomass and NADH fluorescence were monitored online. An *E. coli* strain that constitutively expresses *Vitreoscilla* hemoglobin (VHb) was used and compared with its parent strain. pDNA yields were inversely proportional to the OTR_{max} for both strains and increased more than two-fold in cultures at the lowest OTR_{max} compared with that in aerobic cultures. Expression of VHb increased the specific growth rate at OTR_{max} values of 10, 14 and 30 mmol L^{-1} h^{-1} compared with that of the parent strain. NADH-specific fluorescence decreased in cultures of the engineered strain. The pDNA supercoiled fraction (SCF) was greatest in cultures at an OTR_{max} of 30 mmol L^{-1} h^{-1}, reaching 92.9 % for the wild type strain and 98.7 % for the VHb-expressing strain, while no linearized pDNA was detected. This condition was replicated in a 1 L stirred tank bioreactor, and the results closely resembled those of cultures in MTPs.

Keywords: oxygen transfer rate, plasmid DNA, microbioreactors, *Vitreoscilla* hemoglobin, scale-up/scale-down.

Acknowledgements: The authors acknowledge financial support from Consejo Nacional de Ciencia y Tecnología (grants 256617 and 248926).

1. Introduction.

Intensive cultures for plasmid DNA (pDNA) production by *E. coli* can easily become limited by the availability of oxygen when the oxygen demand exceeds the capacity of oxygen transfer in a bioreactor. Oxygen limitation is highly undesirable in cultures of *E. coli* due to the activation of fermentative pathways and the consequential decrease of biomass formation and accumulation of acidic byproducts and carbon waste. However, for pDNA production, some studies indicated that oxygen limitation may enhance pDNA yield on biomass ($Y_{pDNA/X}$). Notably, Ryan and Parulekar [1] reported that the copy number of a pUC-type plasmid in continuous cultures of *E. coli* increased when oxygen limitation occurred. In cultures at an agitation rate of 300 rpm (leading to dissolved oxygen tension (DOT) between 25 and 0 % air saturation), the copy number was 75 % greater than in cultures at 800 rpm (where DOT was close to 100 % air saturation) [1]. Passarinha et al. [2] compared aerobic (DOT = 60 % air saturation) and microaerobic (DOT = 5 % air saturation) batch cultures of *E. coli* bearing a pUC plasmid. At the end of the culture, $Y_{pDNA/X}$ was 61 % greater than in microaerobic cultures. More recently, Jaén and coworkers [3] reported a 38 % increase in $Y_{pDNA/X}$ in cultures at a DOT of 2 % compared with that of fully aerobic cultures. Therefore, oxygen-limited cultures of *E. coli* could be an interesting option to increase pDNA production. The observed increase of $Y_{pDNA/X}$ may also be related to decreased growth rate that results from decreased energy generation under oxygen-limited conditions. In many cases, $Y_{pDNA/X}$ exhibits an inverse proportionality with the specific growth rate (*v gr*., [4]). However, the oxygen transfer rate of a culture may also play an important role because of the need of a host for energy and biomass generation and a compromise to replicate the plasmid. For instance, Yau [5] performed anaerobic cultures (sparging pure nitrogen to the bioreactor) and found that $Y_{pDNA/X}$ was very similar to that obtained under aerobic conditions, which contrasts with the aforementioned reports of microaerobic cultures.

The topology of pDNA is an important factor for therapeutic activity. The Federal Drug Administration recommends that pDNA for therapeutic applications have a content of at least 80 % of a supercoiled fraction (SCF). It has been demonstrated that the SCF is relevant when transfecting CHO cells for transient recombinant protein expression [6]. DNA supercoiling in *E. coli* depends on at least 4 topoisomerases, of which DNA gyrase and topoisomerase I have the most important activities [7]. The activity of gyrase is sensitive to the ATP/ADP ratio [7] and phosphorylation potential [8]. Anaerobicity affects the activity of these enzymes [9, 10, 11] and consequently affects DNA supercoiling. Accordingly, anaerobiosis has been reported to increase DNA supercoiling. However, the effect of different oxygen limitation levels on pDNA supercoiling has not been described.

In this study, the effect of different levels of oxygen supply, leading to fully aerobic or oxygen-limited conditions, on pDNA yields and SCF was studied. Some of the undesirable effects of oxygen limitation on the physiology of *E. coli* can be attenuated by the expression of *Vitreoscilla* hemoglobin (VHb) [12]. Therefore, this study included a comparison with an engineered *E. coli* strain, in which VHb is constitutively expressed from a chromosomal insertion. The presence of VHb has been shown to improve ATP generation [12] and likely causes changes to the O/P ratio under microaerobic conditions [13]. Therefore, the presence of VHb could influence pDNA supercoiling, because it is expected to alter the ATP/ADP ratio and phosphorylation potential.

2. Methodology

2.1. Strains, plasmid and culture medium

The strains used in this study were derivatives of *Escherichia coli* W3110. In the W3110 *recA*$^-$ strain, the *recA* gene was interrupted by the *cat* (chloramphenicol acetyltransferase) gene. This mutation has been shown to increase plasmid stability and increase the SCF [14]. The W3110 *recA*$^-$ *vgb*$^+$ strain was constructed by inserting the VHb gene (*vgb*) under control of the *trc* promoter as described elsewhere [15]. The *vgb* gene insertion interrupted the *lacI* gene; therefore, the expression of *vgb* is constitutive. Both strains were transformed with the pVAX1 plasmid (Thermo Fisher Scientific, Waltham, MA, USA), a 3 kb high-copy number plasmid that confers kanamycin resistance for selection in *E. coli*. The transformed cells were cultured in Terrific Broth (TB), supplemented with kanamycin sulfate (Sigma, St. Louis, MO, USA) (50 mg L^{-1}) and cryopreserved in 40 % glycerol at -80 °C.

2.2. Culture medium and precultures

All cultures were performed in TB medium (Sigma, St. Louis, MO, USA), which consists of 24 g L^{-1} yeast extract, 20 g L^{-1} tryptone, 2.31 g L^{-1} KH_2PO_4, 12.54 g L^{-1} K_2HPO_4 and 5 g L^{-1} glycerol. The pH was adjusted to 7.4, and the medium was sterilized by autoclaving. Kanamycin sulfate was added to the cold medium at a final concentration of 50 mg L^{-1}. Precultures were prepared using 100 µL of cryopreserved cells to inoculate 10 mL of TB medium and incubated in 250 mL shaker flasks at 37 °C and 350 rpm with a shaking diameter of 50 mm for 12 h. These cultures were used to inoculate the main cultures (initial optical density at 600 nm wavelength of 0.3 AU).

2.3. Cultures in MTPs

MTP cultures were performed using the BioLector system (m2p Labs, Baesweiler, Germany), which allows online measurements of cell growth, DOT, pH and specific fluorescence as indicator of NADH content. Two types of plates were used (round wells (MTP-R48-BOH, Lot 1402, m2p Labs) or Flower plates (MTP-48-BOH, Lot 1515, m2p Labs)), which allow relatively low or high OTRs based on the well geometry. The plates were sealed with a hydrophobic porous rayon sterile sealing film (AeraSeal, Excel Scientific, Victorville, CA, USA). Cultures were performed at 37 °C, 85 % humidity, a shaking diameter of 3 mm. The shaking frequency was as stated for each experiment. Depending on the experiment, the culture volume per well was 800 or 1200 µL. Biomass was monitored by scattered light (λ_{ex} = 620 nm; gain: 20). Fluorescence was used to monitor DOT (λ_{ex} = 520 nm; λ_{em} = 600 nm; gain: 83), pH (λ_{ex} = 485 nm; λ_{em}= 530 nm; gain: 45) and NADH (λ_{ex} = 365 nm; λ_{em} = 460 nm; gain: 60). The culture volumes used were 800, 900, 1500 or 2100 µL, and the agitation rates were 650, 800 or 900 rpm depending on the experiment. All cultures were performed in duplicate.

2.4. Cultures in stirred tank bioreactor (STB)

Cultures in STB were performed in a 1 L BioStat B Plus bioreactor (Sartorius BBI, Melsungen, Germany) with a 600 mL working volume, 200 mL h^{-1} air flow rate and 485 rpm stirring rate. The pH was set at 7.0 and controlled by the automated addition of 15 % NH_4OH. DOT was measured using an optical VisFerm sensor (Hamilton, Reno, NV, USA). The sensor was calibrated by flowing pure N_2 (for 0 % air saturation) or air (for 100 % air saturation) at 1 VVM. Off-gas composition was monitored online using a BlueInOne Ferm (BlueSens, Herten, Germany) gas analyzer.

2.5. Plasmid DNA analysis

Samples for pDNA analysis were collected during the exponential growth phase at the times indicated for each experiment. Samples were centrifuged for 10 min at 13,000 rpm, and the supernatant was discarded. Cell pellets were stored at -20 °C for no more than 1 week. pDNA was isolated using a QIAgen Spin Miniprep kit (Qiagen, Germany) following the manufacturer's instructions. Total double-stranded DNA was measured spectrophotometrically using a NanoDrop One (Thermo Scientific, USA). pDNA samples from cultures at an OTR_{max} *ca.* 10, 30 and 110 mmol L^{-1} h^{-1} were analyzed by agarose gel electrophoresis [16] to determine the DNA topology.

2.5. Offline analysis

The biomass concentration was determined as the dry cell weight for STB cultures. Organic acids were quantified by HPLC using a Bio-Rad Aminex HPX-87H column (Bio-Rad Laboratories Inc., CA, USA) at 50 °C with a 0.4 mL/min flow of 5 mM H_2SO_4 and a UV detector at a wavelength of 210 nm.

2.6. Data analysis.

The initial scattered light and fluorescence intensity data points were subtracted from the measured data, since the accuracy of these measurements are affected by factors such as media background, filling volume or type of microtiter plate [17]. NADH-specific fluorescence was calculated for each data point by dividing the NADH fluorescence value by the corresponding scattered light value. For MTP cultures, the OTR_{max} values were obtained from Funke et al. [18]. For STB cultures, the OTR was calculated from the proper mass balances using the off-gas composition data [3].

3. Results and discussion

3.1. Cultures in MTPs

3.1.1. Aerobic cultures

Cell growth and pDNA yields were studied under fully aerobic conditions. These conditions were achieved by using baffled MTPs and an appropriate liquid volume and agitation speed. The kinetic profiles are shown in Fig. 1. For both strains, aerobic conditions were satisfied, since the DOT was always greater than 15 % air saturation (Fig. 1A). The pH initially decreased to ca. 7.2 (Fig. 1B), and reached the lowest values at the lowest DOT values and then increased along with increasing DOT values, indicating the depletion of the carbon source. The NADH-specific fluorescence for the W3110 *recA*$^-$ *vgb*$^+$ strain was less than its parental strain, but after the main carbon source was depleted, both strains had similar values (Fig. 1C).

It has been shown that the presence of VHb increases the oxygen uptake rate (q_{O2}) [19, 20], which should cause an increase in the oxidation of NADH in the respiratory chain, which may explain the decreased NADH-specific fluorescence detected for the W3110 *recA*$^-$ *vgb*$^+$ strain. This is also in agreement with the hypothesis that increased NADH oxidation rates may increase carbon flux through the tricarboxylic acid cycle, resulting in decreased aerobic acetate accumulation [20, 21]. A decrease in acetate production was also observed for the aerobic cultures shown in Fig. 1, as described in section 3.1.3. The engineered strain displayed a shorter *lag* phase and reached a slightly lower final biomass concentration compared with that of its parent strain (Fig. 1D).

3.1.2. Oxygen-limited cultures

Both strains were cultured under oxygen-limited conditions at four different maximum oxygen transfer capacities, OTR_{max} (10, 14, 30 and 45 mmol L^{-1} h^{-1}). These experiments were aimed at investigating the biological responses to the mass transfer capacity, which is constrained by the bioreactor operation. Fig. 2 shows the culture profiles at the lowest OTR_{max} values that were studied (10 and 14 mmol L^{-1} h^{-1}). DOT was rapidly depleted under both conditions by the parent and engineered strains and remained close to zero during most of the culture (Fig. 2A and 2E). The DOT signal increased nearly 7 h earlier in cultures of the engineered strain at OTR_{max} = 10 mmol L^{-1} h^{-1} compared to that of its parent strain (Fig. 2A). This coincided with a faster change of the pH, probably due to amino acid catabolism [22] (Fig. 2B) and biomass accumulation (Fig. 2D) in the engineered strain, particularly between 30-36 h of culture. However, at OTR_{max} = 14 mmol L^{-1} h^{-1}, DOT profiles were very similar for both strains (Fig. 2E). This suggests that the effect of VHb was more relevant for oxygen consumption at the lowest OTR_{max} values. NADH-specific fluorescence increased during the first 7-7.5 h of culture and then decreased for both strains and OTR_{max} conditions (Fig. 2C and 2G), which coincided with a pH increase (Fig. 2B and 2F). The increase of NADH-specific fluorescence has been predominantly associated with oxidative metabolism, which is the case under aerobic conditions, while the decrease of this signal indicates a deviation to reductive metabolism, such as during oxygen-limited conditions [23]. Due to the complex nature of the culture medium, the changes in NADH-specific fluorescence may also indicate the exhaustion of different influential components in the medium. NADH-specific fluorescence increased until the DOT signal increased for the parent strain and then drastically decreased. In contrast, the increase of NADH-specific fluorescence was reduced for the engineered strain and remained relatively constant during the final phase of the cultures. Although these trends were similar for both OTR_{max} values, the changes were less pronounced in cultures at OTR_{max} = 14 mmol L^{-1} h^{-1} than at 10 mmol L^{-1} h^{-1}. Reduced fluctuations of NADH-specific

fluorescence can be an indicator of a more stable metabolic performance of the engineered strain compared to the parent strain, which is highly desirable for a more robust bioprocesses under environmental fluctuations [24]. Cell growth was markedly reduced when dissolved oxygen was limited in all cases (Fig. 2D, 2H). Despite the presence of VHb, both strains had similar biomass concentrations by the end of the culture (Fig. 2D, 2H).

Fig. 3 shows the culture profiles when OTR_{max} values of 30 and 45 mmol L^{-1} h^{-1} were used. These cultures were considerably shorter than those with lower OTRmax values, while oxygen limitation was observed in all cases (Fig. 3A, 3E). Changes in pH (Fig. 3B, 3F) were less drastic but showed behavior similar to the previously described cultures (Fig. 2B, 2F). In contrast to the cultures with lower OTR_{max} values, NADH-specific fluorescence increased during the aerobic phase of the cultures and remained relatively constant until the exhaustion of the main carbon source (Fig. 3C, 2G), as indicated by the steep increase in DOT (Fig. 3A, 2E). A less fluctuating NADH-specific fluorescence occurred due to a less restricted oxygen availability compared to the cultures shown in Fig.2. Interestingly, NADH-specific fluorescence for the engineered strain was less than that of the parent strain for all OTR_{max} values that were studied, which is consistent with the proposed function of VHb on metabolism [12, 25]. Regarding cell growth, the presence of VHb resulted in shorter *lag* phases, while similar biomass concentrations were observed by the end of the cultures (Fig. 3D, 3H).

3.1.3. Effect of OTR_{max} on organic acid accumulation

Samples at the end of the cultures were analyzed to quantify glycerol and fermentative byproducts. Glycerol was completely exhausted in samples from oxygen-limited cultures. In aerobic cultures, residual glycerol concentrations of 0.03 and 0.53 g L^{-1} were detected for cultures of W3110 *recA*$^{-}$ and W3110 *recA*$^{-}$ *vgb*$^{+}$ strains, respectively. The results of the organic acid analysis are shown in Fig. 4. Neither ethanol nor formate were detected in any sample. Acetate was the main byproduct that was detected (Fig. 4A), followed by succinate (Fig. 4B) and lactate (Fig. 4C). Under fully aerobic conditions, the parent strain accumulated 1.83 ± 0.01 g L^{-1} acetate, which occurred due to overflow metabolism. Interestingly, the engineered strain accumulated only 0.09 ± 0.01 g L^{-1} acetate. VHb efficiently reduced the overflow metabolism of glucose in *E. coli* [20]. Fig. 4A shows that the overflow metabolism of glycerol was also strongly decreased by VHb action. The accumulation of acetate increased with a decrease in oxygen availability. The maximum concentrations of acetate were detected in cultures at OTR_{max} values of 30 and 14 mmol L^{-1} h^{-1} (Fig. 4A) and were significantly reduced for the engineered strain than for the parent strain. Small amounts of succinate (less than 0.25 g L^{-1}) were also detected in aerobic cultures. Succinate

accumulation under microaerobic conditions increased when the OTR_{max} was reduced to reach concentrations close to 1.4 g L^{-1}. The accumulation of succinate under all oxygen limitation conditions that were studied was greater for the engineered strain than for the parent (Fig. 4B). Lactate was not produced in aerobic cultures and was only detected in cultures of the parent strain at OTR_{max} values of 30 and 14 mmol L^{-1} h^{-1} (Fig. 4C). Overall, these results suggest that the presence of VHb results in a greater flux of pyruvate to the tricarboxylic acid (TCA) cycle. The presence of VHb could increase the regeneration of NAD^+ in the electron transport chain, which decreases the need for NAD^+ regeneration by lactate formation. Succinate dehydrogenase is inhibited and fumarate reductase is activated by oxygen limitation [26], which together with a greater flux of pyruvate to the TCA cycle, may explain the greater accumulation of succinate in the engineered strain. Therefore, according to the proposed mechanism of VHb, the engineered cells generated more energy in the electron transport chain than in the parent strain, which is in agreement with the NADH-specific fluorescence. Since no higher biomass or pDNA synthesis were observed, more carbon should have been oxidized to CO_2 and excreted from the cell.

3.1.4. Effect of OTR_{max} on pDNA yields and quality

Fig. 5 depicts the specific growth rates (Fig. 5A) and pDNA yields (Fig. 5B) during the exponential growth phase of fully aerobic cultures and the oxygen-limited phase of microaerobic cultures. pDNA yields were determined in the samples, as shown in Figs. 1-3. Specific growth rates were directly proportional to OTR_{max} (Fig. 5A). This result was expected, since energy generation is greatest under fully aerobic conditions. Oxygen limitation leads to partial oxidation of the carbon source with the subsequent accumulation of fermentative byproducts and substantially reduced ATP generation per mol of carbon. The engineered strain displayed a slightly higher growth rate compared with that of the parent strain at OTR_{max} values of 10, 14 and 30 mmol L^{-1} h^{-1}. This effect is commonly observed in *E. coli* expressing VHb under oxygen limitation [12]. Although growth rate improvements are generally found in most reports using *E. coli* expressing VHb, the VHb gene is plasmid-encoded, which means that hundreds of copies are present. In this study, the engineered strain has only one copy of the gene per cell. However, the effect of VHb was noticeable. At higher OTR_{max} values, the presence of VHb had no effect on the growth rates. The results of aerobic cultures are similar to those reported by Pablos et al. [20] using a high copy number phagemid for VHb expression. Overall, the decrease of OTR_{max} from 110 to 10 mmol L^{-1} h^{-1} caused a 94 % decrease in the growth rate of the parent strain and a 90 % decrease in the growth rate of the engineered strain.

Contrary to the specific growth rate, the pDNA yields on biomass were inversely proportional to OTR_{max} for both strains (Fig. 5B). This demonstrates the utility of oxygen limitation as a simple tool for improving pDNA production processes. The pDNA yield in cultures of the parent strain was 231 % at the lowest OTR_{max} value compared with that in fully aerobic conditions. For the engineered strain, there was a 264 % increase (Fig. 5B). These increases are substantially greater than previously reported results. Specifically, Passarinha and coworkers [2] reported a 61 % increase of $Y_{pDNA/X}$ in cultures at a DOT of 5 % compared with cultures at a DOT of 60 %. Ryan and Parulekar [1] reported a 67 % increase in the plasmid copy number when *E. coli* was cultured in a 0.8 L working volume bioreactor using a stirring speed of 200 rpm (oxygen limitation occurred during most of the culture) compared with agitation at 800 rpm. More recently, Jaén and coworkers [3] reported a 38 % increase of $Y_{pDNA/X}$ in cultures at a DOT of 2 % compared with fully aerobic cultures. Interestingly, in all studied conditions, $Y_{pDNA/X}$ was always lower for the engineered strain than for the parent strain. Pablos et al. [20] reported that the W3110 strain doubled its pDNA content under aerobic conditions as a result of VHb expression. This result contrasts with the results reported in Fig. 5B. However, the previous study used a minimal medium for cell growth, and the *vgb* gene was coded in the plasmid, which greatly increased its copy number. These factors can cause different cellular responses. Overall, under the conditions studied in the present report, it can be concluded that the expression of VHb under oxygen-limited conditions leads to increased biomass synthesis rates and lower organic acid accumulation rather than increasing the pDNA content in cells.

The topology of pDNA is an important parameter that is used to assess culture performance. Three conditions were selected for analyzing pDNA topology, which included aerobic conditions, intermediate OTR_{max} (30 mmol L^{-1} h^{-1}) and lowest OTR_{max} (10 mmol L^{-1} h^{-1}). The results are shown in Table 1. There was no correlation between the topology and OTR_{max}. Under fully aerobic conditions, the supercoiled pDNA fraction (SCF) was the same for both strains. Small fractions of nicked pDNA were detected for the parent strain but not for the engineered strain. Nicked pDNA was also detected in relatively low proportions for both strains. The highest SCF for both strains was obtained at an OTR_{max} of 30 mmol L^{-1} h^{-1}. For this particular condition, the SCF for the engineered strain was close to 100 %. No linear pDNA was detected for any strain, and the fraction of nicked pDNA was less than that found under fully aerobic conditions. The most heterogeneous pDNA was produced at the lowest OTR_{max}, where linear and nicked pDNA were detected and the SCF was less than 90 % for both strains.

In general, the SCF was greater than 80 % for all conditions and strains tested, which indicates that pDNA quality was not compromised by oxygen limitation. Differences in the SCF between strains were only found at OTR_{max} = 30 mmol L^{-1} h^{-1} and were small. If ATP availability affected the supercoiling process, it is possible that under these oxygen-limited conditions, increased ATP generation caused by VHb was indicated by a slightly greater SCF. The effect of VHb under fully aerobic conditions and very low OTR_{max} was irrelevant. Increasing the gene copy number in the engineered strain may be useful to improve these results.

Overall, the described results indicate that OTR_{max} = 30 mmol L^{-1} h^{-1} is the optimal condition to produce pDNA under oxygen limitation since $Y_{pDNA/X}$ was reasonably increased compared with that of aerobic cultures, and the SCF was also the greatest.

3.2. Cultures in a 1 L stirred tank bioreactor

The optimal OTR_{max} that was used in MTPs was evaluated in a standard lab-scale STB. This was performed to evaluate whether microbioreactors can be used to scale-up pDNA production to stirred bioreactors. The W3110 *recA*$^-$ strain was used, due to its greater $Y_{pDNA/X}$ in comparison with that of the engineered strain. Fig. 6 shows the culture profile in a 1 L bioreactor at an OTR_{max} = 30 mmol L^{-1} h^{-1}. Precultures were developed with the same methods as those that were used for MTP cultures. Oxygen depletion required an additional 1 h than in MTPs, which was likely due to a longer *lag* phase (Fig. 6A). Dissolved oxygen was depleted approximately 4 h after inoculation, and the OTR reached the maximum value (30.2 ± 1.2 mmol L^{-1} h^{-1} during *ca.* 4.5-11 h) shortly thereafter (Fig. 6B). During the oxygen-limited period, the specific growth rate in STB cultures was 0.10 ± 0.01 h^{-1}, which is very similar to that in MTP cultures (0.09 ± 0.00 h^{-1}). The increase of DOT at 11.2 h indicated the exhaustion of the main carbon source, followed by a temporal DOT decrease, indicating the partial consumption of metabolic byproducts. The cessation of respiratory activity was clearly observed from the rapid DOT increase and OTR decrease at approximately 12.5 h (Fig. 6B).

To compare MTP and STB cultures under the most similar conditions, samples were collected after the cells grew for 3 h under oxygen limitation. Organic acids in the supernatant and pDNA were analyzed. Table 2 shows the amounts of organic acids that were detected. The amounts of acetate, succinate and lactate that were detected in STB cultures (Table 2) were very similar to those in the MTP cultures (Fig. 4). However, the amount of lactate in STB cultures was much greater than that in MTP cultures. In contrast with MTP cultures, some formate was detected in STB cultures. This indicated that factors like the lack of pH control in the MTP's as well as a

higher osmolality due to the addition of MOPS, influenced the distribution of carbon fluxes at the pyruvate node. Formate hydrogenlyase is an enzyme that converts formate to CO_2 and H_2 at pH values of approximately 6.5 [27]. This condition was achieved shortly before the time of collecting MTP culture samples but was not observed in the STB cultures, where the pH was controlled. Therefore, it is possible that the formate that was produced was efficiently transformed to CO_2 and H_2 only in the MTP cultures, where no formate accumulation was detected.

The biomass was analyzed using different methods in MTP and STB cultures. Therefore, $Y_{pDNA/X}$ is not an appropriate parameter for comparison. Instead, the pDNA titer was compared. Table 3 reports the pDNA titer in the samples that were collected from MTP cultures and the corresponding STB cultures. The amount of pDNA in MTP cultures was *ca*. 20 % lower than that of STB cultures. Although the culture profiles and byproduct accumulation were very similar in both culture systems, unlike the MTPs, the STB was operated at constant pH. It has been reported that pH values and pH fluctuations during cultures strongly impact the observed $Y_{pDNA/X}$ [28, 29]. It is therefore probable that the constant pH conditions in STB cultures contributed to a greater $Y_{pDNA/X}$ compared with that in MTP cultures. The SCF of the pDNA that was produced in STB cultures was also assessed and was 94.1 ± 1.0 %. This value is very similar to that obtained in MTP cultures (92.9 ± 0.9 %).

4. Conclusions

Oxygen limitation was evaluated as a strategy to increase pDNA yields in cultures of wild type and engineered *E. coli* expressing *Vitreoscilla* hemoglobin using microbioreactors. It was demonstrated that oxygen limitation efficiently enhanced pDNA yields in both strains, although the engineered strain displayed reduced pDNA yields and increased growth rates. Even though the smaller OTR_{max} resulted in greater pDNA topological heterogeneity, the SCF was greater than 80 % in all studied conditions. The selected OTR_{max} condition of 30 mmol L^{-1} h^{-1} in a 1 L STB led to very similar pDNA titers and, in general, a very similar kinetic behavior compared to those in MTP cultures. Therefore, the suitability of MTPs for assessing oxygen-limited cultures and their utility as general predictors during the scale-up process to a lab-scale STB was also demonstrated.

Abbreviations

d_0 Shaking diameter (mm)

DOT Dissolved oxygen tension (% air saturation)

MTP Microtiter plate

n shaking frequency (rpm)

NADH Reduced nicotinamide adenine dinucleotide

OTR Oxygen transfer rate (mmol L^{-1} h^{-1})

OTR_{max} Maximum oxygen transfer rate (mmol L^{-1} h^{-1})

STB Stirred tank bioreactor

V_L Volume of the liquid phase (μL)

References

[1] W. Ryan, S.J. Parulekar, Effects of culture conditions on plasmid stability and production of a plasmid-encoded protein in batch and continuous cultures of *Escherichia coli* JM103[pUC8]. Ann NY Acad Sci. 589 (1990) 91-110.

[2] L.A. Passarinha, M.M Diogo, J.A. Queiroz, G.A. Monteiro, L.P. Fonseca, D.M.F. Prazeres, Production of ColE1 type plasmid by *Escherichia coli* DH5α cultured under nonselective conditions. J Microbiol Biotecnol. 16 (2006) 20-24.

[3] K.E. Jaén, R. Olivares, J.C. Sigala, A.R. Lara, Effects of oxygen availability on plasmid DNA production by *Escherichia coli*. BMC Biotechnol. 17 (2017) 60.

[4] M. Wunderlich, H. Taymaz-Nikerel, G. Gosset, O.T. Ramírez, A.R. Lara, Effect of growth rate on plasmid DNA production and metabolic performance of engineered *Escherichia coli* strains. J Biosci Bioeng. 17 (2014): 336-342.

[5] S. Y. Yau, An Investigation on *E. coli* host strain influences and strategies to improve supercoiled plasmid DNA production for gene therapy and vaccination. PhD Thesis. University College London. 2010.

[6] A. Dhanoya, B.M Chain, E. Keshavarz-Moore, The impact of DNA topology on polyplex uptake and transfection efficiency in mammalian cells. J Biotechnol. 155 (2011) 377–386.

[7] K. Drlica, Control of bacterial DNA supercoiling. Mol Microbiol. 6 (1992) 425-433.

[8] M. van Workum, S.J.M. van Dooren, N. Oldenburg, D. Molenaar, P.R. Jensen, J.L. Snoep, H.V. Westerhoff, DNA supercoiling depends on the phosphorylation potential in *Escherichia coli*. Mol Microbiol. 20 (1996) 351-360.

[9] S. Cortassa, M.A. Aon, Altered topoisomerase activities may be involved in the regulation of DNA supercoiling in aerobic-anaerobic transitions in *Escherichia coli*. Mol Cell Biochem. 126 (1993) 115-124.

[10] L.S. Hsieh, R.M. Burger, K. Drlica, Bacterial DNA supercoiling and [ATP]/[ADP]. Changes associated with a transition to anaerobic growth. J Mol Biol. 219 (1991) 443-450.

[11] C. J. Dorman, G. C. Barr, N. N. Bhriain, C. F. Higgins, DNA supercoiling and the anaerobic and growth phase regulation of *tonB* gene expression. J Bacteriol. 170 (1988) 2816-2826.

[12] B.C. Stark, K.R. Pagilla, K.L. Dikshit, Recent applications of *Vitreoscilla* hemoglobin technology in bioproduct synthesis and bioremediation. Appl Microbiol Biotechnol. 99 (2015) 1627-1636.

[13] T.E. Pablos, R. Olivares, J.C. Sigala, O.T. Ramírez, A.R. Lara, Toward efficient microaerobic processes using engineered *Escherichia coli* W3110 strains. Eng Life Sci. 16 (2016) 588-597.

[14] G.M. Borja, E. Meza, G. Gosset, O.T. Ramírez, A.R. Lara, Engineering *E. coli* to increase plasmid DNA production in high cell-density cultivations in batch mode. Microb Cell Fact. 11 (2012) 132.

[15] A.R. Lara, K.E. Jaén, M. Mühlmann, J.C. Sigala, L. Regestein, J. Büchs, Characterization of endogenous and reduced promoters for oxygen-limited processes using *Escherichia coli*. ACS Synth Biol. 6 (2017) 344-356.

[16] S. Hassan, E. Keshavarz-Moore, J Ward, A cell engineering strategy to enhance supercoiled plasmid DNA production for gene therapy. Biotechnol Bioeng. 113 (2016) 2064-2071.

[17] M. Kunze, M. Roth, E. Gartz, J. Büchs, Pitfalls in optical on-line monitoring for high-throughput screening of microbial systems. Microb Cell Fact. 13 (2014) 53.

[18] M. Funke, S. Diederichs, F. Kensy, C. Müller, J. Büchs, The baffled microtiter plate: Increased oxygen transfer and improved online monitoring in small scale fermentations. Biotechnol Bioeng. 103 (2009) 1118−1128.

[19] P.S. Tsai, M. Nägeli, J.E. Bailey, Intracellular expression of *Vitreoscilla* hemoglobin modifies microaerobic *Escherichia coli* metabolism through elevated concentration and specific activity of cytochrome *o*. Biotechnol Bioeng. 49 (1996) 151–160.

[20] T.E. Pablos, J.C. Sigala, S. Le Borgne, A.R. Lara, Aerobic expression of *Vitreoscilla* hemoglobin efficiently reduces overflow metabolism in *Escherichia coli*. Biotechnol J. 9 (2014) 791-799.

[21] G.N. Vemuri, M.A. Eiteman, E. Altman, Increased recombinant protein production in *Escherichia coli* strains with overexpressed water-forming NADH oxidase and a deleted ArcA regulatory protein. Biotechnol Bioeng. 94 (2006) 538–542.

[22] G. Sezonov, D. Joseleau-Petit, R. D'Ari, *Escherichia coli* physiology in Luria-Bertani broth. J Bacteriol. 189 (2007) 8746-8749.

[23] S. Assawajaruwan, F. Kuon, M. Funke, B. Hitzmann, Feedback control based on NADH fluorescence intensity for *Saccharomyces cerevisiae* cultivations. Bioresour Bioprocess. 5 (2018) 24.

[24] A.L Heins, D. Weuster-Botz, Population heterogeneity in microbial bioprocesses: origin, analysis, mechanisms, and future perspectives. Bioproc Biosyst Eng. 41(2018) 889-916.

[25] P.S. Tsai, G. Rao, J.E. Bailey, Improvement of *Escherichia coli* microaerobic oxygen metabolism by *Vitreoscilla* Hemoglobin: New insights from NAD(P)H fluorescence and culture redox potential. Biotechnol Bioeng. 47 (1995) 347-354.

[26] B. Ward, Bacterial energy metabolism, in: Y.W. Tang, M. Sussmann, D. Liu, I. Poxton (Eds.), Molecular Medical Microbiology, Academic Press, London, 2015, pp. 201-233.

[27] A. Yoshida, T. Nishimura, H. Kawaguchi, M. Inui, H. Yukawa, Enhanced hydrogen production from formic acid by formate hydrogen lyase-overexpressing *Escherichia coli* strains. Appl Environ Microbiol. 71 (2005) 6762-6768.

[28] T. Cortés, N. Flores, F. Bolívar, A.R. Lara, O.T. Ramírez, Physiological effects of pH gradients on *Escherichia coli* during plasmid DNA production. Biotechnol Bioeng. 113 (2016) 598-611.

[29] C.M. Ongkudon, R. Pickering, D. Webster, M.K. Danquah, Cultivation of *E. coli* carrying a plasmid-based Measles vaccine construct (4.2 kbp pcDNA3F) employing medium optimisation and pH-temperature induction techniques. Microb Cell Fact. 10 (2011) 16.

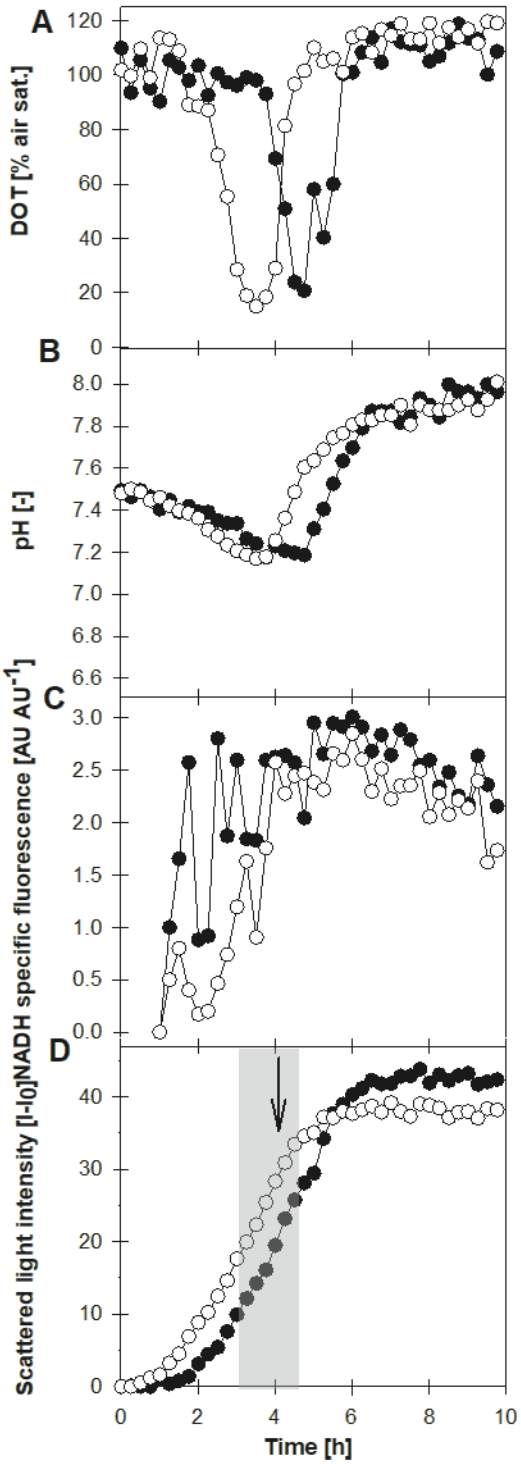

Figure 1. Aerobic cultures of *E. coli* W3110 *recA*$^-$ (filled circles) and W3110 *recA*$^-$ *vgb*$^+$ (empty circles). Online monitoring of DOT (A), pH (B), NADH specific fluorescence (C), and cell growth (D) data are shown. Culture conditions: 48-well FlowerPlate®, V_L = 800 µL, n = 1500 rpm, d_0 = 3 mm (OTR_{max} ca. 110 mmol L^{-1} h^{-1}), TB medium. The shaded area denotes the period considered for calculation of the specific growth rate. The vertical arrow indicates the sampling point for pDNA analyses.

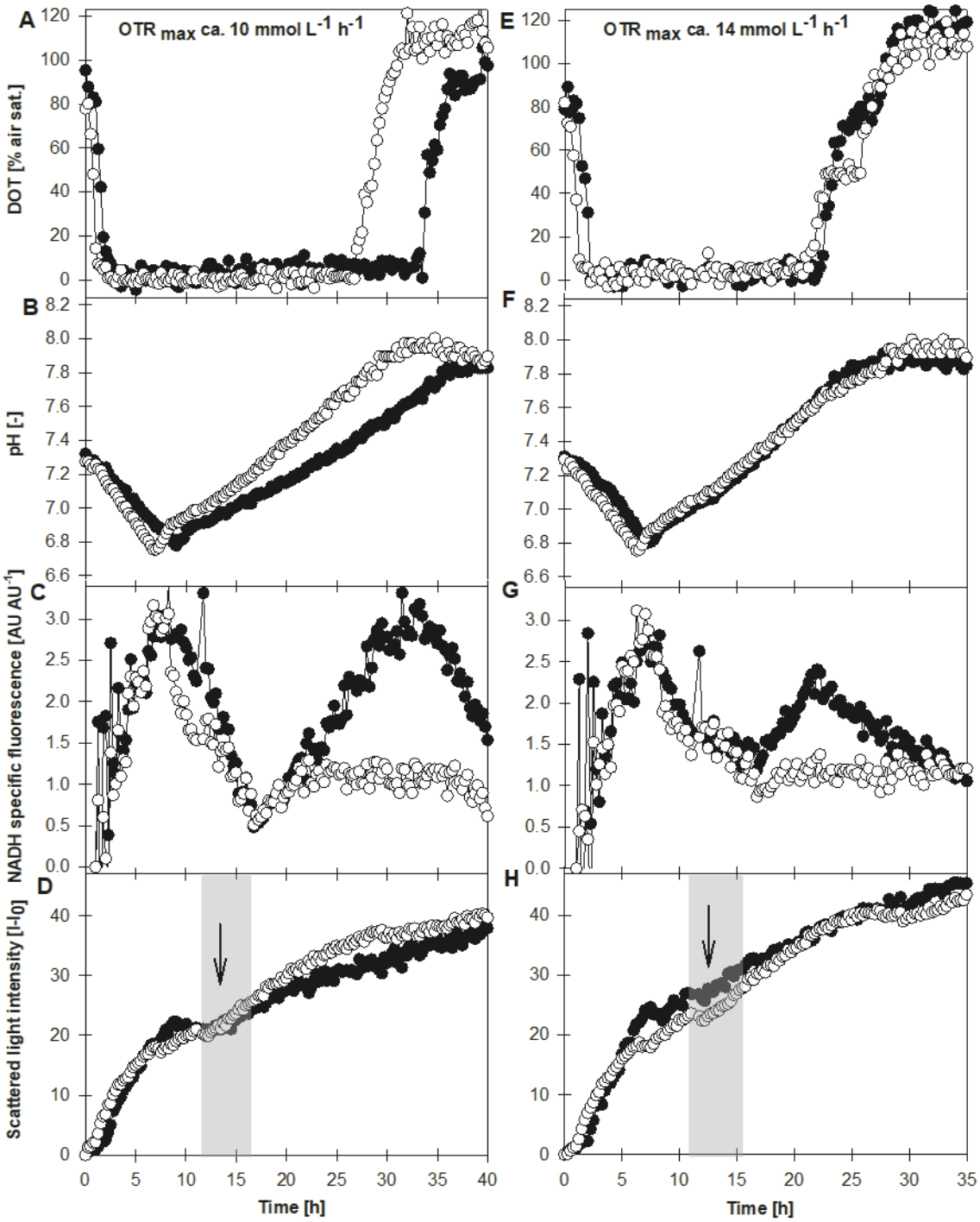

Figure 2 Oxygen-limited cultures of *E. coli* W3110 *recA*$^-$ (filled circles) and W3110 *recA*$^-$ *vgb*$^+$ (empty circles). Online monitoring of DOT (A, E), pH (B, F), NADH specific fluorescence (C, G) and cell growth by scattered light (D, H) data are shown. Culture conditions: 48-well round well plate, d_0 = 3 mm, V_L = 2100 µL (for OTR_{max} ca. 10 mmol L^{-1} h^{-1}) or 1500 µL (for OTR_{max} ca. 14 mmol L^{-1} h^{-1}), n = 800 rpm, TB medium. The shaded area denotes the period considered for calculation of the specific growth rate. The vertical arrows show the sampling points for pDNA analyses.

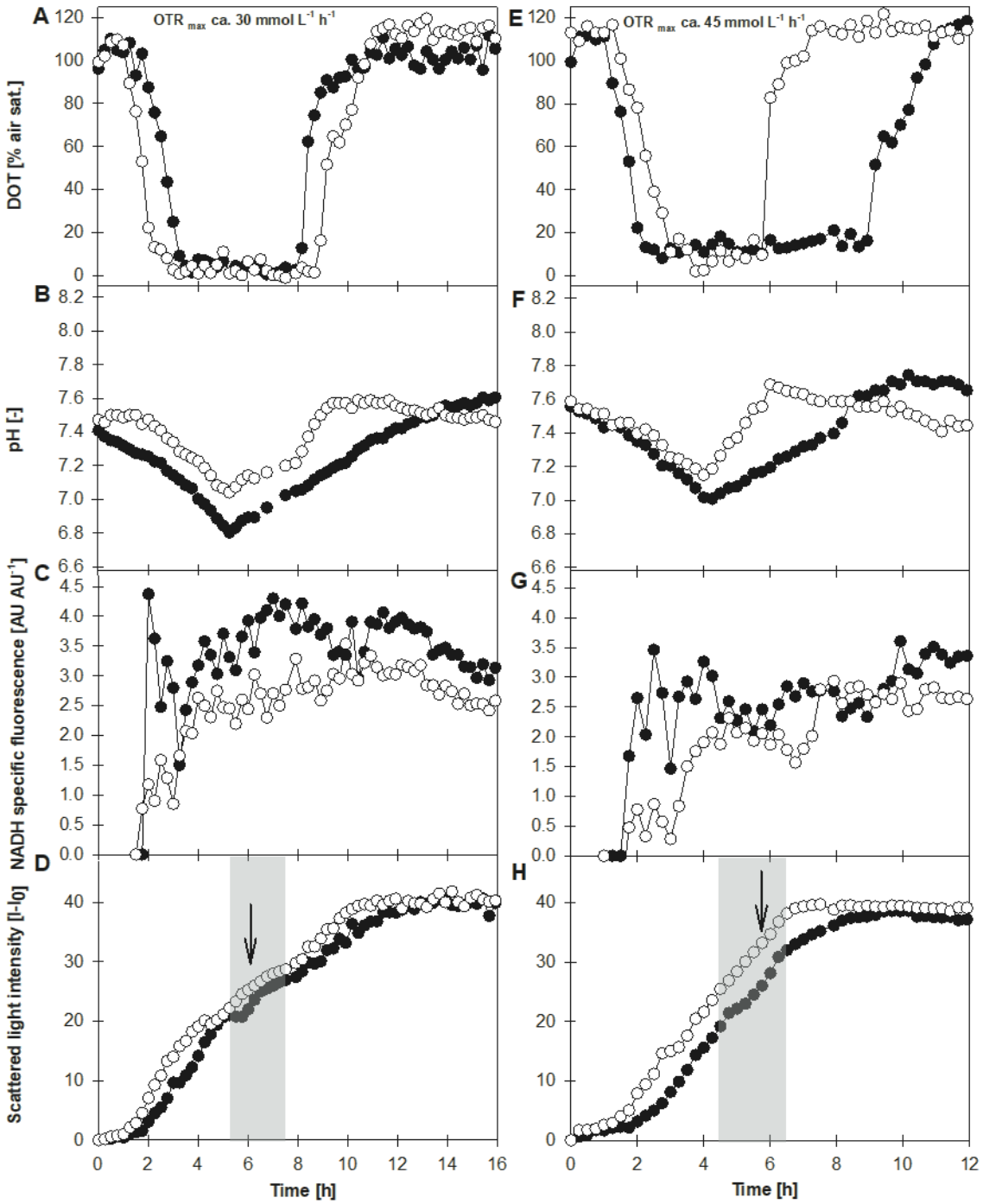

Figure 3 Oxygen-limited cultures of *E. coli* W3110 *recA*$^-$ (filled circles) and W3110 *recA*$^-$ *vgb*$^+$ (empty circles). Online monitoring of DOT (A, E), pH (B, F), NADH specific fluorescence (C, G) and cell growth by scattered light (D, H) data are shown. Culture conditions: 48-well FlowerPlate®, d_0 = 3 mm, V_L = 1500 µL (for OTR_{max} ca. 30 mmol L^{-1} h^{-1}) or 800 µL (for OTR_{max} ca. 45 mmol L^{-1} h^{-1}), n = 900 rpm, TB medium. The shaded area denotes the period considered for calculation of the specific growth rate. The vertical arrows show the sampling points for pDNA analyses.

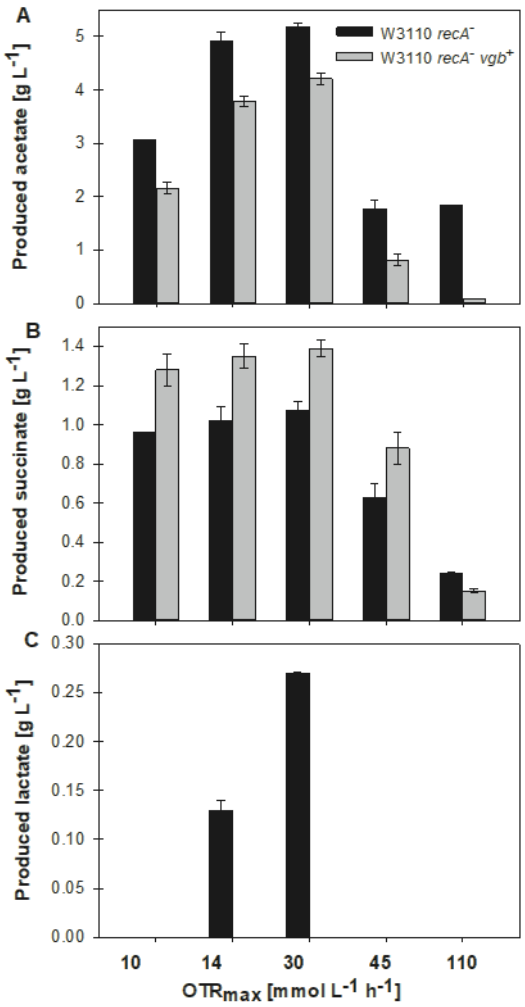

Figure 4. Effect of the maximum oxygen transfer capacity, OTR_{max} on the accumulation of acetate (A), succinate (B), and lactate (C). Error bars show the deviation between duplicate experiments.

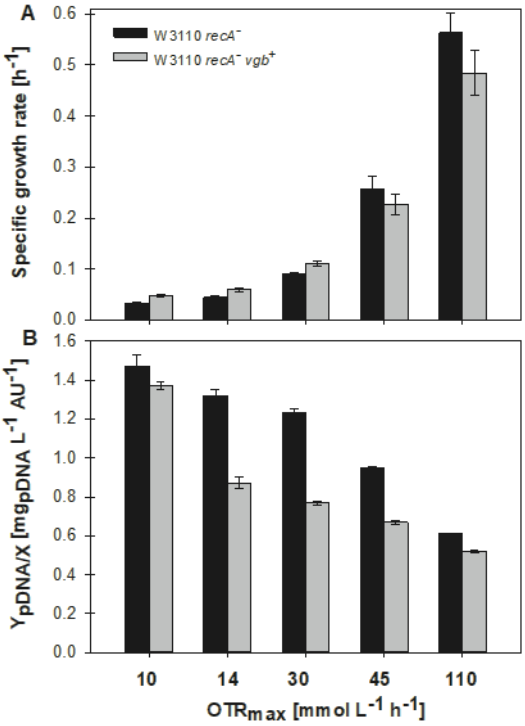

Figure 5. Effect of OTR_{max} on specific growth rates (A) and pDNA yields on biomass (B). Data are average of duplicate cultures. Vertical lines show the experimental deviation between duplicates.

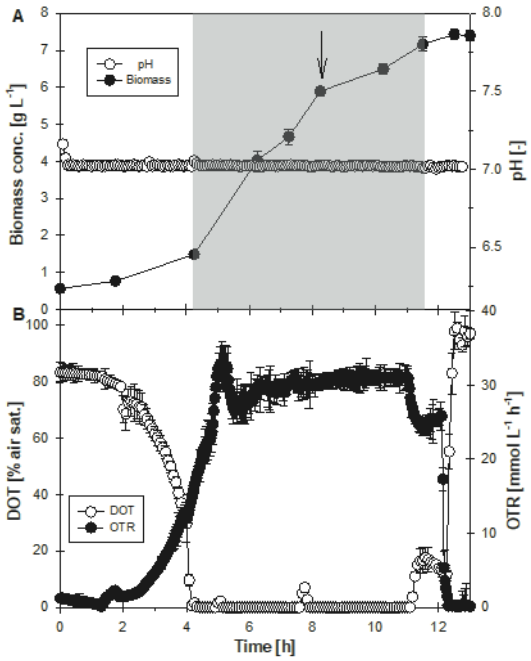

Figure 6. Oxygen-limited cultures of *E. coli* W3110 *recA*$^{-}$ in a stirred tank reactor operated at an OTR_{max} *ca.* 30 mmol L^{-1} h^{-1}. Biomass growth and pH (A), DOT and OTR (B) data are shown. Culture conditions: 600 mL working volume, air flow rate 0.3 vvm, stirring rate of 485 rpm and pH 7.0. TB medium. The shaded area denotes the period considered for calculation of the specific growth rate. The vertical arrow shows the sampling point for pDNA analysis.

Table 1. Topological analysis of the pDNA obtained under selected OTR_{max} conditions.

OTR_{max} [mmol L^{-1} h^{-1}]	**Strain**	**Nicked [%]**	**Linear [%]**	**Supercoiled [%]**
110	W3110 *recA*$^-$	7.4	0	92.6 ± 1.7
	W3110 *recA*$^-$ *vgb*$^+$	5.3	2.1	93.0 ± 2.1
30	W3110 *recA*$^-$	7.2	0	92.9 ± 0.9
	W3110 *recA*$^-$ *vgb*$^+$	1.3	0	98.7 ± 1.3
10	W3110 *recA*$^-$	6.1	5.3	88.6 ± 1.3
	W3110 *recA*$^-$ *vgb*$^+$	9.4	4.4	86.0 ± 1.0

Table 2. Concentration of fermentative by-products in the sample taken after 3 h under oxygen limitation in cultures performed in a 1 L STB at an OTR_{max} of *ca*. 30 mmol L^{-1} h^{-1}.

Organic acid	**Concentration [g L^{-1}]**
Acetate	5.11 ± 0.01
Succinate	1.74 ± 0.11
Lactate	0.97 ± 0.06
Formate	0.85 ± 0.12

Table 3. pDNA concentration and SFC after 3 h under oxygen limitation in cultures at an OTR_{max} of *ca*. 30 mmol L^{-1} h^{-1} performed in MTPs and 1 L STB.

	MTP	**1 L STB**
pDNA conc. [mg L^{-1}]	29.0 ± 0.4	35.0 ± 1.5