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RESEARCH ARTICLE

BIOTECHNOLOGY PROGRESS

Comprehensive analysis of metabolic sensitivity of 1,4-butanediol producing *Escherichia coli* toward substrate and oxygen availability

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Abstract

Nowadays, chemical production of 1,4-butanediol is supplemented by biotechnological processes using a genetically modified *Escherichia coli* strain, which is an industrial showcase of successful application of metabolic engineering. However, large scale bioprocess performance can be affected by presence of physical and chemical gradients in bioreactors which are a consequence of imperfect mixing and limited oxygen transfer. Hence, upscaling comes along with local and time dependent fluctuations of cultivation conditions. This study emphasizes on scale-up related effects of microbial 1,4-butanediol production by comprehensive bioprocess characterization in lab scale. Due to metabolic network constraints 1,4-butanediol formation takes place under oxygen limited microaerobic conditions, which can be hardly realized in large scale bioreactor. The purpose of this study was to assess the extent to which substrate and oxygen availability influence the productivity. It was found, that the substrate specific product yield and the production rate are higher under substrate excess than under substrate limitation. Furthermore, the level of oxygen supply within microaerobic conditions revealed strong effects on product and by-product formation. Under strong oxygen deprivation nearly 30% of the consumed carbon is converted into 1,4-butanediol, whereas an increase in oxygen supply results in 1,4-butanediol reduction of 77%. Strikingly, increasing oxygen availability leads to strong increase of main by-product acetate as well as doubled carbon dioxide formation. The study provides clear evidence that scale-up of microaerobic bioprocesses constitute a substantial challenge. Although oxygen is strictly required for product formation, the data give clear evidence that terms of anaerobic and especially aerobic conditions strongly interfere with 1,4-butanediol production.

KEYWORDS

1,4-butanediol, bioreactor inhomogeneity, microaerobic production process, oxygen deprivation

1 | INTRODUCTION

The chemical industry currently undergoes a paradigm change and progress is achieved to replace fossil based resources by renewable ones.[1-3] In recent studies a set of chemical compounds have been identified that can serve as intermediates for bridging biological and chemical processes.[4,5] In the list of C4-compounds 1,4-butanediol (BDO) is listed which can serve as chemical synthon besides its direct application as alcohol monomer compound for polyester synthesis.[6,7]

Bio-based production of diols has been achieved in several microbial systems, such as 2,3-butanediol in *Saccharomyces cerevisiae*,[8] *Bacillus subtilis*,[9] *Bacillus licheniformis*[10] and many more, 1,3-butanediol in *Escherichia coli*,[11] 1,3-propanediol in *Klebsiella pneumoniae*,[12] *Clostridium butyricum*, *Lactobacillus brevis*, and more,[13] and most successfully 1,4-butanediol in *Escherichia coli*.[14-16] Because of its commercial interest as bio-based monomer for polyester synthesis the economic evaluation clearly shows, that BDO production requires large scale bioreactor operation to meet market demand as well as reasonable process economics.[14] This goes along with increasing bioreactor inhomogeneity, that is, spatio-temporal changes of environmental conditions for certain volume elements. Such effects can be simulated under lab scale conditions following the scale-down approach with compartmented bioreactor systems, providing frequent oscillation in the environmental conditions while parts of the culture pass the additional compartments.[17,18] Hence robustness studies toward oscillating environmental conditions are continuously gaining higher relevance. Such studies provided detailed insight into microbial production of for example valinomycin in *E. coli*,[19] L-lysine in *Corynebacterium glutamicum*,[20] preproinsulin in *E. coli*[21] or even the efficiency of plasmid DNA production in general.[22]

Depending on the microbial system and the redox state of substrate and product, microbial production processes can be distinguished. Many of the current production processes are operated under aerobic conditions,[23] but an increasing number of processes were developed which enable product formation under nonaerated or anaerobic conditions,[24] for example, isobutanol,[25] succinate[26] as well as propionate.[27] The decisive factor for successful production processes under oxygen limitation is a suitable substrate/product combination. Such a substrate/product pair is characterized by meeting the energy demand for substrate to product conversion under oxygen limitation including transport mechanisms, possible product excretion and feasibility in commercial scale.[28]

In this sense BDO represents an interesting example, since the metabolic pathway from substrate glucose to BDO requires microaerobic conditions. Oxygen deprived conditions are obligatory to enable the network to regenerate sufficient reduced redox equivalents in form of NADH to fuel the reduction of the substrate glucose to the more reduced product BDO.

The heterologous biosynthetic pathway to BDO in *E. coli* ECKh-422 is illustrated in Figure 1. It is branching from the TCA cycle either from alpha-ketoglutarate or from succinyl-CoA (Figure 1). The route from succinyl-CoA requires a reduction to succinyl semialdehyde catalyzed by succinate semialdehyde dehydrogenase (from *Porphyromonas gingivalis*, *sucD*) in the first step. This reaction consumes NADH and is consequently more expensive for the cell than the decarboxylation from alpha-ketoglutarate via alpha-ketoglutarate decarboxylase (from *Mycobacterium bovis*, *sucA*) to succinyl semialdehyde. Hence, the route via decarboxylation of alpha-ketoglutarate is thermodynamically more favorable and seem to be preferred and used up to 95%.[15]

Succinyl semialdehyde is further reduced by 4-hydroxybutyrate dehydrogenase (from *P. gingivalis*, *4hbd*) into 4-hydroxybutyrate (GHB). The following step is catalyzed by the 4-hydroxybutyryl-CoA transferase (from *P. gingivalis*, *cat2*) consuming one molecule acetyl-CoA and producing one molecule acetate and 4-hydroxybutyryl-CoA. Subsequently two consecutive reduction steps lead to 4-hydroxybutyraldehyde and

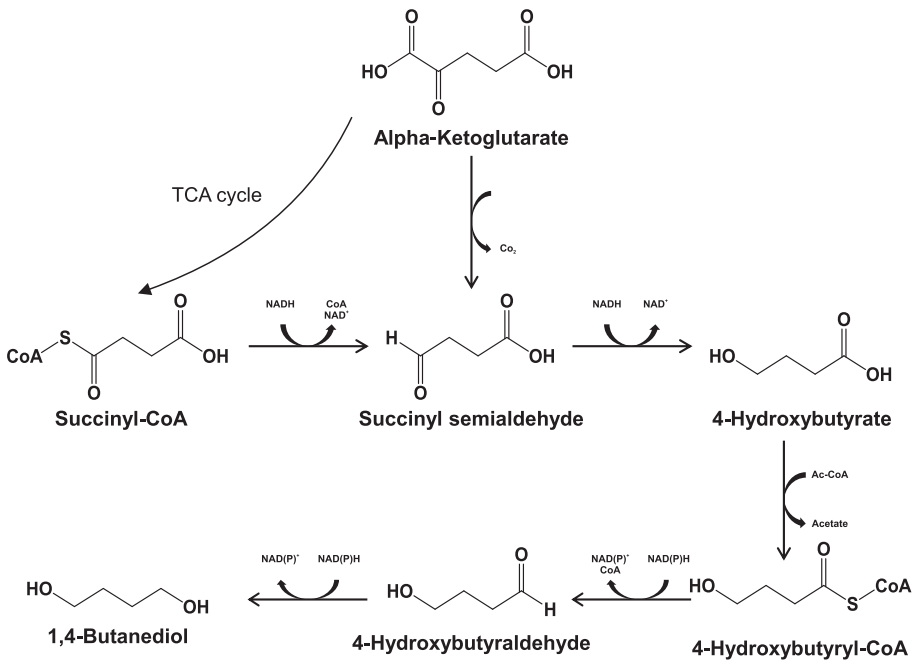

FIGURE 1 Heterologous BDO pathway in *Escherichia coli* ECKh-422

finally to the target product BDO. The first reduction is done by 4-hydroxybutyryl-CoA reductase (from *Clostridium beijerinckii*, *025B*) and the last step in the pathway is done by native alcohol dehydrogenase of *E. coli*.[15]

Most studies in the field of microaerobic BDO production with *E. coli* have only focused on higher BDO productivity obtained by strain engineering at laboratory scale and potential issues of scale-up related changes of oxygen and substrate concentrations did not receive attention so far. Having this in mind, the study provides a comprehensive bioprocess characterization of the BDO producing strain *E. coli* ECKh-422 at different levels of substrate and oxygen supply. Cultivation experiments in batch and fed-batch mode were extensively characterized based on product and by-product formation with closed carbon balance as well as volumetric productivity and product yield. It was found, that microaerobic BDO production with *E. coli* ECKh-422 severely suffers from reduced performance under too low, but especially under too high oxygen supply. For the first time, the study put emphasis on bioprocess development and implications for scale up of microaerobic processes which proved to show additional complexity due to the strict coupling of a certain level of microaerobic conditions to product formation. Therefore, this study makes a major contribution to research on microaerobic industrial processes by demonstrating the strong influence of small changes in cultivation environment on bioprocess efficiency.

2 | METHODS

2.1 | Bacterial strain and plasmids

2.1.1 | *Escherichia coli*

ECKh-422 is a 1,4-butanediol producing *E. coli* strain of Genomatica (San Diego, CA). This strain was designed from the host strain *E. coli* MG1655*lacI*^{Q} by introducing the two plasmids pZS*13S-sucCD-sucD-4hbd/sucA and pZE23-025B-Cat2 which harbor the genes for the heterologous biosynthetic BDO pathway. Additionally, several gene deletions were introduced (*ΔadhE*, *ΔpflB*, *ΔldhA*, *ΔlpdA*::*Klebsiella pneumonia*, *Δmdh*, and *ΔarcA*) so that the metabolic network is tailored for microaerobic production conditions.[15]

2.2 | Cultivation conditions

Cultivations were performed in parallel bioreactor system of DASGIP® (Eppendorf, Hamburg, Germany) with a working volume of 1 L in M9 minimal medium (6.78 g l^{-1} Na_2HPO_4, 3.0 g l^{-1} KH_2PO_4, 0.5 g l^{-1} NaCl, 2.0 g l^{-1} NH_4Cl, 1.0 g l^{-1} $[NH_4]_2SO_4$, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$). The temperature was held at 37°C and the pH was titrated to seven by addition of 2 M NH_3 and 30% H_3PO_4. The cells were grown in an initial aerobic growth phase with dissolved oxygen regulated at 30% until an OD_{600} of 10, where the culture was induced with 0.25 mM IPTG. One hour later the culture was switched to microaerobic conditions for BDO production. Therefore the base was changed to 2 M Na_2CO_3 and stirring was set constant at 700 rpm and a gassing rate of 1, 2 or 6 sl hr^{-1} was adjusted with OTR_{max} of 7.4, 9.8, and 20.9 mmol l^{-1} hr^{-1}, respectively.

2.3 | Analytical procedures

Cell dry weight was determined from 2 ml cell suspension in dried reaction tubes (> 48 hr, 80°C). After centrifugation at 16,060*g* for 10 min, the supernatant was discarded and the cell pellet was washed with 0.9% (wt/vol) NaCl. Subsequently, the cell pellet was dried at 80°C for 48 hr following the gravimetric determination of CDW.

Product, substrate and by-products were quantified in culture supernatant by HPLC. The culture supernatant was separated from cells by centrifugation at 16,060*g* for 10 min and filtrated through a cellulose-acetate syringe filter (0.2 μm, DIA-Nielsen, Düren, Germany). The compounds were separated by HPLC (Agilent 1,100 and 1,200 Infinity, Agilent Technologies, Santa Clara) with isocratic method using 0.1 M H_2SO_4 at a flow rate of 0.6 ml min^{-1} (80°C) or 0.8 ml min^{-1} (45°C) applying cation exchange chromatography (Organic acid resin HPLC-column, Metab-AAC, 300 x 7.8 mm, CS Chromatographie Service, Langerwehe, Germany). Detection was carried out with UV-light at 215 nm or refraction index at 191 nm.

2.4 | Untargeted qualitative metabolite profiling

For identification of unknown by-products which cause a gap in the carbon balance an untargeted qualitative metabolite profiling of the culture supernatant was performed according to the method described by Evans et al.[29]. The culture supernatant was extracted with methanol under shaking for 2 min to precipitate protein and dissociate small molecules bound to protein. After a centrifugation step the resulting extracts (supernatants) were measured with four different UPLC-MS/MS methods on Waters ACQUITY UPLC (Waters Corporation, Milford) and a Q-Exactive™ high resolution/accuracy mass spectrometer (Thermo Fisher Scientific, Waltham) with H-ESI source and orbitrap mass analyzer operated at 35,000 mass resolution (cf. supplementary information for method details).

3 | RESULTS AND DISCUSSION

3.1 | Influence of substrate availability

The BDO production strain was cultivated under glucose limited and excess conditions. All cultivations were started with an initial aerobic batch phase for biomass growth. Afterward a switch to microaerobic conditions led to steady decrease of growth rate until non growing conditions are reached with a maximal total biomass of 7 and 6.8 g for glucose excess and limiting conditions, respectively (Figure 2a). To establish microaerobic conditions an aeration rate of 2 sl hr^{-1} was chosen which was initially derived from Yim, et al.[15] and used for initial bioreactor cultivation experiments

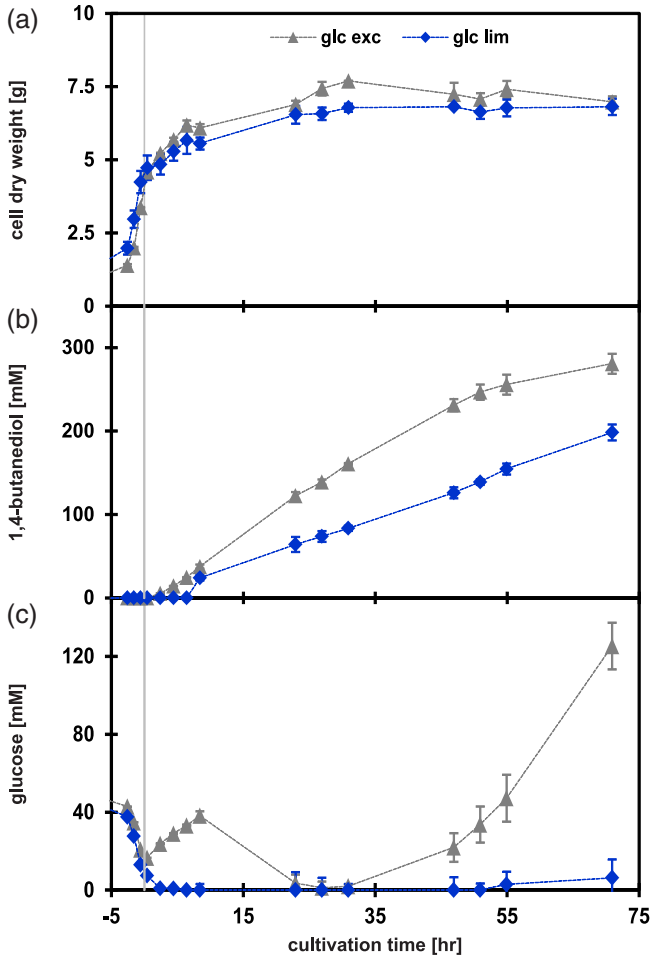

FIGURE 2 Process data of *Escherichia coli* ECKh-422 under substrate excess in comparison to substrate limitation. Biomass, BDO, and glucose concentration are compared between substrate excess and substrate limitation. The vertical line at time point 0 hr marks the beginning of the microaerobic production phase ($OTR_{max,glc\ lim}$ = 12.4 mmol l^{-1} hr^{-1}, $OTR_{max,glc\ exc}$ = 10.3 mmol l^{-1} hr^{-1}) after an initial aerobic growth phase. Error bars correspond to minimum and maximum values of biological duplicates, n_b = 2

(data not shown). For limiting glucose conditions a feed rate of F(t) = 0.01 ml hr^{-1} * t + 2.4 ml was used, because weak growth was still assumed for the microaerobic phase according to Yim, et al.[15] Feed rate for glucose surplus conditions was set to F(t) = 0.03 ml hr^{-1}* t + 3 ml. Nevertheless, during cultivation under glucose surplus conditions in the mid of production phase glucose concentration fell below 20 mM and increased again in the later phase up to the final value of 120 mM (Figure 2c). This indicates that glucose consumption rate is higher under oxygen limitation than during aerobic growth phase,[30,31] but seems to stay in a constant range over process time under microaerobic conditions. Although, the glucose concentration showed a strong decrease between 23 and 31 hr glucose excess can be assumed throughout the experiment, since the lowest residual glucose was still measured at 2–3 mM in the cultivation supernatant. In terms of process economics the linear feed rate was not optimal for the microaerobic production phase due to glucose overfeed beginning from 47 hr, but the strain behavior was not negatively affected by higher glucose concentrations. In the glucose limiting experiment the glucose concentration was below level of detection, so that limiting conditions were ensured.

During the microaerobic phase BDO production is observed showing almost linear increase of BDO until the cultivation was stopped. A final BDO concentration of 281 and 198 mM were found for the glucose excess and limiting process after 71 hr production phase, respectively (Figure 2b). The comparative analysis clearly showed that the glucose excess conditions provided better process performance in terms of volumetric product formation with 0.08 g_P g_{CDW}^{-1} hr^{-1} ± 0.01 for glucose excess and 0.05 g_P g_{CDW}^{-1} hr^{-1} ± 0.00 for glucose limitation. Furthermore a higher substrate related product yield was measured under glucose excess $Y_{P/S}$ = 0.22 g_P g_{CDW}^{-1} ± 0.01 compared to substrate limited conditions $Y_{P/S}$ = 0.18 g_P g_{CDW}^{-1} ± 0.00 (cf. Table 1).

Unless many other examples for *E. coli* showing that glucose limited conditions are better suited for production processes,[19,32] this example demonstrates that for microaerobic BDO formation glucose surplus is superior. Strikingly, this result has a major effect on the scale-up strategy and on the bioreactor inhomogeneities that need to be considered. The results clearly indicate that it is not necessary to supply glucose under limited conditions and no glucose limited fed-batch process is required. As a consequence substrate inhomogeneity need not to be considered for large scale bioreactor operation and scale-down bioreactor experiments can focus on other parameters showing inhomogeneities, such as microaerobic oxygen supply.

3.2 | Influence of oxygen supply under microaerobic conditions

Based on metabolic robustness of the BDO strain toward substrate surplus conditions, the following experiments were performed under substrate excess. Here, the supply with oxygen was further investigated, since microaerobic conditions are expected to inherently generate bioreactor inhomogeneity for oxygen concentration. As for any other limiting substrate, oxygen cannot be supplied in a homogeneous manner in large scale bioreactor. Hence, it is of high interest to investigate the dependency of the BDO formation with respect to the amount of oxygen supplied. The bioprocess performance data of different conditions are summarized in Table 1.

The BDO producing strain was cultivated in the presence of glucose excess with the initial aerobic growth phase, followed by the switch to microaerobic conditions with aeration ranging from 1 to 6 sl hr^{-1} air (Figure 3). All cultivations showed a dissolved oxygen of 0% in this phase and were therefore stated as microaerobic. Notice that 2 sl hr^{-1} was used as standard reference value for the experiment with changed substrate supply yielding final BDO concentration of 274 mM with $Y_{P/S}$ = 0.21 ± 0.02 g_P g_{CDW}^{-1} under glucose excess, accompanied by 38 mM 4-hydroxybutyric acid, 283 mM acetate, 223 mM ethanol as relevant by-products.

TABLE 1 Overview of product formation and substrate uptake under different conditions

	Glc exc (n_b = 2)	Glc lim (n_b = 2)	1 sl hr^{-1} (Glc exc, n_b = 3)	2 sl hr^{-1} (Glc exc, n_b = 3)	6 sl hr^{-1} (Glc exc, n_b = 3)
q_P (g_P g_{CDW}^{-1} hr^{-1})	0.08 ± 0.01	0.05 ± 0.00	0.07 ± 0.01	0.07 ± 0.01	0.02 ± 0.00
q_S (g_S g_{CDW}^{-1} hr^{-1})	0.31 ± 0.00	0.23 ± 0.00	0.28 ± 0.01	0.30 ± 0.02	0.18 ± 0.08
$Y_{P/S}$ (g_P g_S^{-1})	0.22 ± 0.01	0.18 ± 0.00	0.24 ± 0.03	0.21 ± 0.02	0.11 ± 0.05
$CDW_{51\ hr}$ (g)	7.08 ± 0.2	6.64 ± 0.1	5.98 ± 0.5	7.49 ± 0.6	7.44 ± 0.3
$BDO_{51\ hr}$ (g)	28.05 ± 0.5	14.54 ± 0.2	20.74 ± 3.7	25.75 ± 3.6	6.18 ± 1.7
$Acetate_{51\ hr}$ (g)	15.3 ± 1.3	13.28 ± 0.1	6.59 ± 0.6	16.21 ± 1.3	28.27 ± 4.7
$Pyruvate_{51\ hr}$ (g)	3.44 ± 0.6	0.72 ± 0.3	4.83 ± 0.7	3.25 ± 0.6	0.49 ± 0.1
$Ethanol_{51\ hr}$ (g)	15.87 ± 0.1	13.2 ± 0.2	6.73 ± 2.6	12.17 ± 5.4	2.03 ± 1.5
γ-$Hydroxybutyrate_{51\ hr}$ (g)	–	–	4.27 ± 1.4	4.92 ± 1.1	1.64 ± 0.8
$Alanine_{51\ hr}$ (g)	1.89 ± 0.1	–	4.44 ± 1.2	2.97 ± 1.5	0.99 ± 0.5
OTR_{max} (mmol L^{-1} hr^{-1})	10.3 ± 0.2	12.4 ± 0.94	7.4 ± 1.4	9.8 ± 0.67	20.9 ± 2

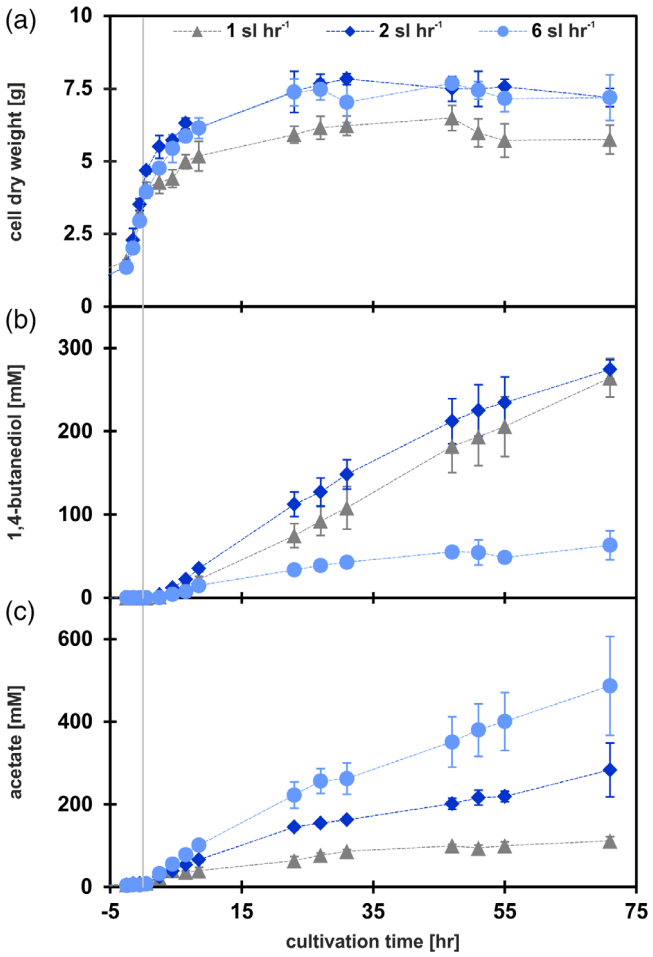

FIGURE 3 Process data of *Escherichia coli* ECKh-422 under different microaerobic conditions. Extracellular data under different gassing rates (1 sL hr^{-1} with OTR_{max} = 7.4 mmol l^{-1} hr^{-1}, 2 sl hr^{-1} with OTR_{max} = 9.8 mmol l^{-1} hr^{-1}, 6 sl hr^{-1} with OTR_{max} = 20.9 mmol l^{-1} hr^{-1}) are illustrated. The dissolved oxygen was zero in all cases and all cultivations were performed under substrate excess. The vertical dashed line at time point 0 hr marks the beginning of the microaerobic production phase after an initial aerobic growth phase. Error bars correspond to standard deviation of biological triplicates, n_b = 3

The initial aerobic growth pattern is very similar for the three conditions, and the microaerobic phase with aeration rate of 1, 2, and 6 sl hr^{-1} started with similar biomass of ~4 g (Figure 3a). In the first 8 hr of the microaerobic phase a weak growth is ascertainable, finally leading to a non-growing state. Despite of this, a remarkable negative production phenotype is observed for 6 sl hr^{-1} (Figure 3b). Compared to the reference cultivation with 2 sl hr^{-1}, the higher supply of oxygen reduces final product titer to 63 mM (–77%). Further reduction of the aeration rate to 1 sl hr^{-1} shows comparable BDO titer at the end, but improved substrate specific product yield up to 0.24 ± 0.03 g_P g_S^{-1} considering the lower biomass. The main by-product acetate was further reduced from 283 to 111 mM (–61%) during cultivations with 2 and 1 sl hr^{-1}. The highest oxygen supply leads to the highest acetate formation with 487 mM (Figure 3c). Other studies investigating *E. coli* in scale-down models under oxygen oscillations showed growth reduction up to 30%[21] and considerable by-product formation, especially acetate accumulation.[33,34] The data shown here indicate that most of the acetate seems to be formed by native acetate metabolism and is no direct consequence of the BDO pathway. The results clearly reveal that too high oxygen supply leads to less BDO formation accompanied by higher acetate accumulation.

The final concentration of BDO as well as its share of the carbon balance increased with decreasing oxygen supply (Figure 4). Obviously, a certain level of oxygen deprivation is required for efficient channeling of carbon into the product pathway. While pyruvate and alanine concentration and their respective share of carbon balance show slightly decreasing changes with increasing oxygen supply, a very drastic increase is observed for acetate and CO_2 with higher oxygen transfer rate (Figure 4). In the competition for reducing equivalents between the BDO formation pathway and respiration, higher aeration rates seem to favor respiratory reoxidation of NADH, finally leading to higher CO_2 formation rates concomitantly reducing BDO formation. Other studies revealed that higher dCO_2 concentrations support growth reduction and acetate accumulation in *E. coli*.[35,36] Hence, it might be possible, that at least in the cultivation with 6 sl hr^{-1} aeration high carbon

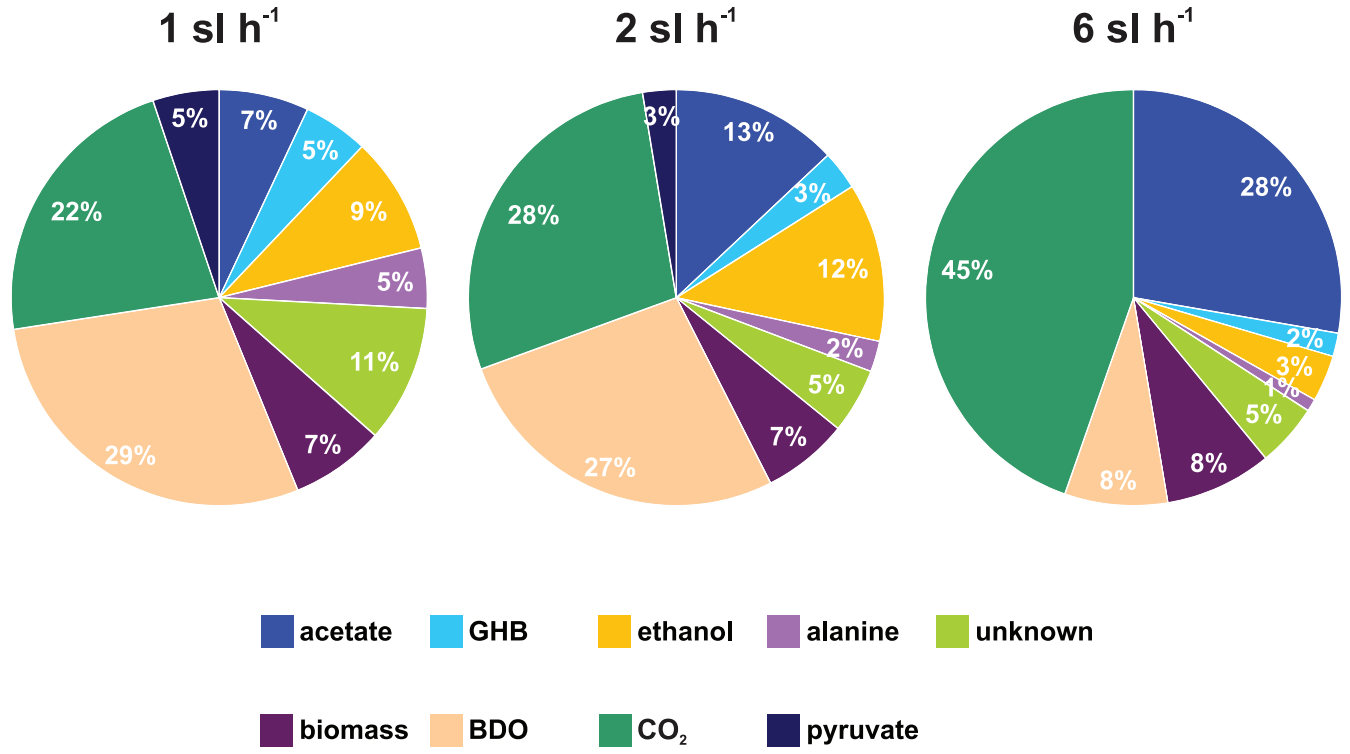

FIGURE 4 Carbon balances of BDO production processes under different oxygen supply. Carbon balances represent the share of carbon in cultivation products after 51 hr microaerobic production phase (1 sl hr^{-1} with OTR_{max} = 7.4 mmol l^{-1} hr^{-1}, 2 sl hr^{-1} with OTR_{max} = 9.8 mmol l^{-1} hr^{-1}, 6 sl hr^{-1} with OTR_{max} = 20.9 mmol l^{-1} hr^{-1}). Mean values are calculated from biological triplicates, n_b = 3

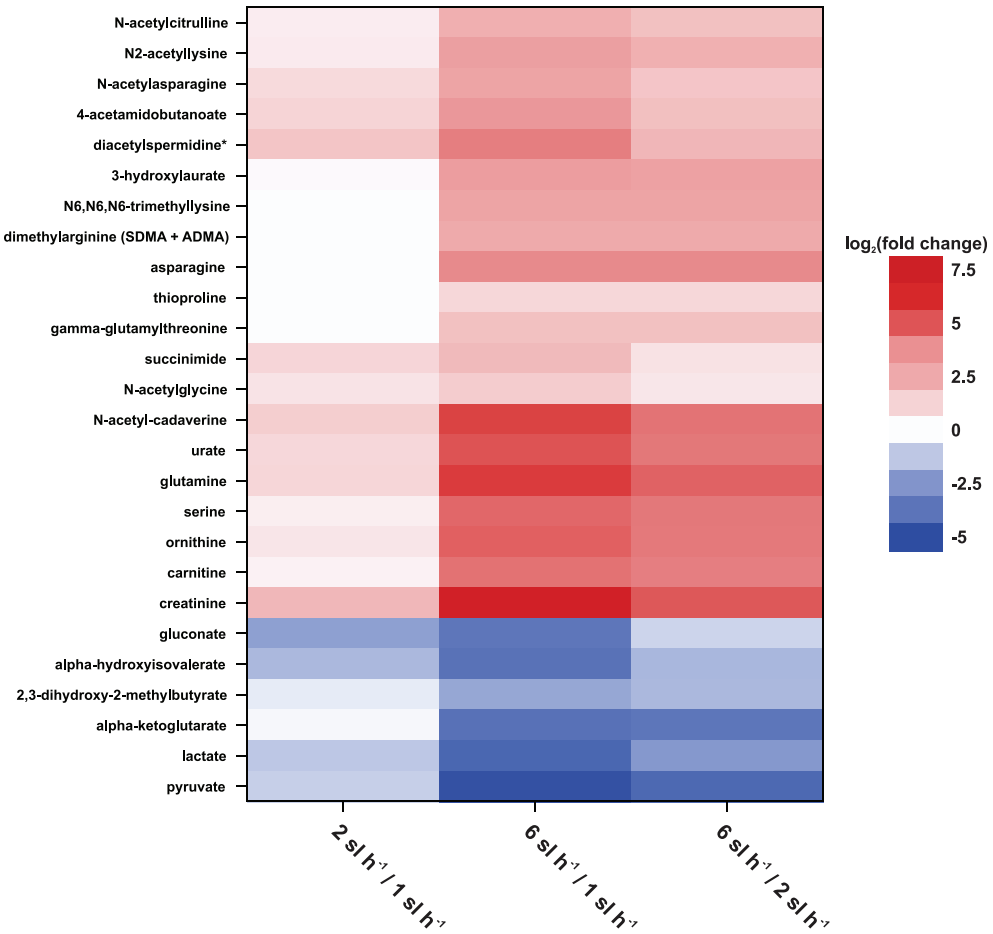

FIGURE 5 Relative comparison of secondary by-products under different oxygen supply. The heatmap represents the fold changes of the 26 most significantly (global adjusted $p < .05$) changed secondary by-products of *E. coli* ECKh-422 under 1, 2, and 6 sl hr^{-1} aeration (1 sl hr^{-1} with OTR_{max} = 7.4 mmol l^{-1} hr^{-1}, 2 sl hr^{-1} with OTR_{max} = 9.8 mmol l^{-1} hr^{-1}, 6 sl hr^{-1} with OTR_{max} = 20.9 mmol l^{-1} hr^{-1}). Fold-changes were calculated from biological triplicates, n_b = 3

dioxide formation can lead to slightly increasing dCO_2 accompanied by stronger acetate accumulation. Higher by-product formation of acetate might be also a consequence of overflow metabolism and reduced acetyl-CoA regeneration,[37] which is not utilized in the heterologous pathway at lower BDO formation. Additionally, the redox state of the cell could be affected by the deletion of regulator protein ArcA, which is part of the aerobic respiratory control two-component system (ArcA/B) and involved in controlling the switch between respiration and fermentation.[30] Deletion of *arcA* is used to minimize acetate formation, because usually it would divert carbon into the acetate metabolism by repressing TCA cycle enzymes to prevent further NADH accumulation caused by higher TCA cycle flux.[38] Nevertheless, increasing acetate formation is observed with increasing oxygen supply. Presumably, the reoxidation of enhanced NADH levels is more effective in the BDO pathway than in respiratory chain under oxygen limited conditions.

Furthermore, ethanol concentration is more than three to fourfold higher for 1 and 2 sl hr^{-1} in comparison to 6 sl hr^{-1}. This could be the effect of native alcohol dehydrogenase activity in *E. coli* which is higher under oxygen limited conditions.[39] However, *E. coli* ECKh-422 is specified as *adhE* mutant and *adhE* is encoding for the alcohol dehydrogenase, which is mainly responsible for ethanol generation in *E. coli*. Strikingly, in all cultivations ethanol was determined, which might be related to the activity of other alcohol dehydrogenases which are present in the *E. coli* strain during the product formation phase.[40] To identify the responsible alcohol dehydrogenase gene in order to reduce ethanol by-product formation, a gene deletion study for other alcohol dehydrogenases could be an option. Furthermore alcohol dehydrogenases are able to reduce several substrates and are also responsible for the conversion of 4-hydroxybutyraldehyde into BDO (cf. Figure 1).

The carbon balances show a gap in the range of 5–11% of carbon, which is not covered by routine analytics. Therefore an untargeted metabolite profiling of the culture supernatant was done to identify unknown compounds and compare the metabolite spectrum.

In total, 211 metabolites were positively identified in the culture supernatants of *E. coli* ECKh-422. Significant metabolite differences between the experiments with different aeration rates were determined by performing global students *t*-test with $p < .05$ as significance criterion. For the 26 most significant metabolites (global adjusted $p < .05$) the fold-changes are illustrated as log2 values in the heatmap shown in Figure 5. Many metabolites are involved in amino acid synthesis or fatty acid synthesis. The comparison of the cultivation with 1 and 2 sl hr^{-1} revealed only weak differences in the by-product spectrum, whereas the cultivation with 6 sl hr^{-1} clearly showed more changes compared to 1 and 2 sl hr^{-1}. In summary, the gap in the carbon balance can be associated to a pattern of different metabolites and is quite unlikely to be represented by one or two missing single by-products only. It can be observed, that metabolites increase from 1 to 6 sl hr^{-1}. A few exceptions are pyruvate, lactate, gluconate, alpha-ketoglutarate, alpha-hydroxyisovalerate, and 2,3-dihydroxy-2-methylbutyrate, which decrease from 1 to 6 sl hr^{-1}. In general, the relative comparison of the metabolites can confirm cultivation data. The experiments with lower aeration rate (1 and 2 sl hr^{-1}) already showed a similar pattern in terms of biomass, BDO and CO_2 formation, while higher aeration rate (6 sl hr^{-1}) resulted in strong differences in product range especially the massive decrease in BDO production.

4 | CONCLUSIONS

The results demonstrate the importance of oxygen limiting conditions for the *E. coli* BDO strain used in this study. *E. coli* ECKh-422 serves as a prototype for an industrial BDO production strain. Regarding the substrate supply, it was shown that substrate excess is the best choice for the highest productivity, so that potential gradient formation resulting from substrate limited fed-batch operation do not have to be considered for scale-up. On the contrary, changes in oxygen availability revealed strong impact on product formation. The metabolic network of the BDO production strain is tailored for optimal functionality during microaerobic conditions[15] (cf. Figure 1). This work shows that any deviation from the optimal oxygen deprivation toward higher oxygen supply leads to much reduced BDO product formation and high by-product formation, that is, mainly acetate and carbon dioxide. Microaerobic conditions were tested under different degrees of oxygen deprivation, where in all cases no dissolved oxygen concentration was measurable. Aeration rates of 1, 2, and 6 sl hr^{-1} were investigated and a reduction of the final BDO concentration up to 77% was observed with the highest aeration rate. Furthermore, the by-product range is strongly influenced by the oxygen supply (cf. Figure 4). However, scale-up of the best microaerobic conditions with 1 sl hr^{-1} from lab scale to pilot and production scale bear challenges due to the increasing inhomogeneity, since homogeneous microaerobic oxygen supply can be hardly achieved. It can be speculated that bioreactor zones in the proximity of the aeration device and bottom impeller providing higher oxygen transfer rate must be kept very small, since a strong negative effect associated with higher respiration coupled to reduced BDO formation might be expected. This study provided reasonable estimation about the most relevant conditions to investigate in terms of BDO process scale-up, namely glucose excess conditions and varying oxygen transfer setup still providing microaerobic conditions. Future activities need to investigate BDO formation using scale-down bioreactor setups coupled with omics profiling of the intracellular metabolic phenotype. This will provide more detailed insight about alternative pathway routes and metabolic response under oscillatory cultivation conditions mimicking large scale operation in order to reveal new targets for metabolic engineering.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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