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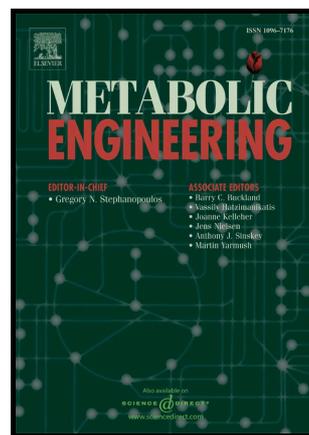
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## Author's Accepted Manuscript

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## Harnessing the respiration machinery for high-yield production of chemicals in metabolically engineered *Lactococcus lactis*

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### Abstract

When modifying the metabolism of living organisms with the aim of achieving biosynthesis of useful compounds, it is essential to ensure that it is possible to achieve overall redox balance. We propose a generalized strategy for this, based on fine-tuning of respiration. The strategy was applied on metabolically engineered *Lactococcus lactis* strains to optimize the production of acetoin and (*R,R*)-2,3-butanediol (R-BDO). In the absence of an external electron acceptor, a surplus of two NADH per acetoin molecule is produced. We found that a fully activated respiration was able to efficiently regenerate NAD<sup>+</sup>, and a high titer of 371 mM (32 g/L) of acetoin was obtained with a yield of 82% of the theoretical maximum. Subsequently, we extended the metabolic pathway from acetoin to R-BDO by introducing the butanediol dehydrogenase gene from *Bacillus subtilis*. Since

one mole of NADH is consumed when acetoin is converted into R-BDO per mole, only the excess of NADH needs to be oxidized via respiration. Either by fine-tuning the respiration capacity or by using a dual-phase fermentation approach involving a switch from fully respiratory to non-respiratory conditions, we obtained 361 mM (32 g/L) R-BDO with a yield of 81% or 365 mM (33 g/L) with a yield of 82%, respectively. These results demonstrate the great potential in using finely-tuned respiration machineries for bio-production.

## Keywords

Respiration capacity; Hemin; *Lactococcus lactis*; Acetoin; (*R,R*)-2,3-butanediol

## 1. Introduction

When selecting a microorganism to be used as a cell factory for production of a particular compound, there are many factors that need to be taken into consideration, e.g., the robustness, the metabolic flexibility and the ability to grow on cheap feedstocks (Lee and Kim, 2015; Nielsen and Keasling, 2016; Stephanopoulos, 2007). It is also important to ensure that production of the desired compound enables redox balance. The importance of this has been demonstrated for various engineered microorganisms, e.g., *Escherichia coli* producing 1-butanol or organic acids (Shen et al., 2011; Kim et al., 2015), *Saccharomyces cerevisiae* producing D-lactic acid (Baek et al., 2016), *Lactococcus lactis* producing (*S,S*)-2,3-butanediol (S-BDO) (Liu et al., 2016a), and *Corynebacterium glutamicum* producing L-valine (Hasegawa et al., 2013). The redox balance is readily attained for the compounds mentioned above without involving an external electron acceptor. However, this is not always possible and an external electron acceptor is needed for the cells to regenerate NAD<sup>+</sup> for the biosynthesis of more oxidized chemicals. Alternatively, various strategies can be used, such as the co-production of different compounds (Gaspar et al., 2011), the simultaneous utilization of different substrates (Wei et al., 2013), the use of transhydrogenase (Choi et al., 2014) and NADH oxidase (NOX) (Liu et al., 2016b). The problems associated with these

strategies are that they are not easily generalized, and they could complicate the engineering strategy or downstream processing for product recovery. Although the NOX, which relies on oxygen as an electron acceptor, has been widely used for regenerating NAD<sup>+</sup> for producing compounds like acetoin, *meso*-2,3-butanediol (m-BDO) and (*R,R*)-2,3-butanediol (R-BDO) in *Bacillus subtilis* and *S. cerevisiae* (Kim and Hahn, 2015; Zhang et al., 2014), it is difficult to precisely control the NOX activity. In addition, a too high activity would drain most of the available NADH and thus reduce the yield of BDO. Currently, most processes developed for BDO production rely on keeping the oxygen tension low to prevent this problem (Biswas et al., 2012; Fu et al., 2014; Li et al., 2010; Li et al., 2015; Lian et al., 2014; Wang et al., 2012; Xu et al., 2014). However, controlling the oxygen tension can be challenging, especially in large-scale fermenters (Zhu et al., 2011). Therefore, there is an urgent need for more robust approaches for fine-tuning the amount of reducing power available.

Respiration capable microorganisms often regulate the NADH/NAD<sup>+</sup> ratio via respiration. When NADH is oxidized into NAD<sup>+</sup>, its electrons are usually transferred to oxygen while protons are pumped across the cell membrane. The resulting proton gradient can then drive ATP formation via oxidative phosphorylation as well as various transport processes (Lane, 2010). Therefore, tampering with the respiration capacity could potentially affect both the NADH/NAD<sup>+</sup> and the ATP/ADP ratio, as demonstrated for *E. coli* by Zhu et al. (Zhu et al., 2011), which could have negative effects on biomass accumulation and growth, and eventually on productivity. For this reason, it would be an advantage to work with a microorganism, which has less coupling between NADH oxidation and ATP production, e.g., *Lactococcus lactis*. *L. lactis* is a lactic acid bacterium, which normally relies on a fermentative metabolism, but it can respire when hemin, an essential cofactor of cytochrome oxidase, is present (Koeblmann et al., 2008; Tachon et al., 2010). Still most of the ATP is generated via substrate level phosphorylation even under respiratory conditions (Koeblmann et al., 2008;

Garrigues et al., 2006). This characteristic indicates that it might be possible to use respiration to oxidize surplus NADH without interfering with the cellular ATP/ADP ratio and thereby growth.

In the current study, we test this hypothesis and modulate respiration by changing the hemin concentration. Focus is on the compounds acetoin and R-BDO, which differ in the amount of reducing power needed for their formation. As a chassis for producing these compounds, we used a metabolically engineered *L. lactis*, where all the main NADH-consuming competitive pathways were eliminated, including three lactate dehydrogenase homologs (LDH), the alcohol dehydrogenase (AdhE), two butanediol dehydrogenases (ButBA), the soluble NADH oxidase (NoxE), and the phosphotransacetylase (PTA). We demonstrate that high-yield production of the two compounds is achieved, either by fine-tuning respiration or by using a dual phase fermentation approach involving a switch from fully-active respiration to anaerobic fermentation. The strategy developed appears robust, and is useful for adjusting the reducing power available.

## 2. Materials and methods

### 2.1. Strains and plasmids

*L. lactis* subsp. *cremoris* MG1363 (Gasson, 1983) and its derivatives were used for the studies.

Strain Ace001 is a derivative of *L. lactis* MG1363, where the genes encoding LDH, PTA, AdhE and ButBA have been inactivated (Liu et al., 2016b). For deleting various genes in *L. lactis*, the plasmid pCS1966 (Solem et al., 2008) was used. The plasmid pTD6 (Solem et al., 2013) was used to express butanediol dehydrogenase BsBdh from *B. subtilis*. The plasmid proGFP was used to express a redox sensitive green fluorescent protein (roGFP), roGFP1-R12 (Cannon and Remington, 2006) in order to assess the intracellular oxidative stress of *L. lactis*. All the constructed strains and plasmids are listed in Table 1.

### 2.2. Cultivation conditions

*L. lactis* MG1363 and its derivatives were cultured in a modified version of the defined SA medium (Jensen and Hammer, 1993) supplemented with different amounts of glucose at 30 °C. The medium was modified by adding 0.2 % (w/v) yeast extract (YE). When needed, 5 µg/ml of tetracycline and chloramphenicol were included in the medium. When needed, hemin was added to the medium to a final concentration of 0.2-10 µg/ml. Growth and fermentation were carried out in either shake flasks or bioreactors. For shake flask culturing, 250-ml flasks containing 30 ml of medium were used, and the shaking speed applied was 200 rpm. The bioreactors (Sartorius Biostat Q) with a 500 ml working volume were used with a stirring speed of 200 rpm and a constant aeration rate of 1.0 vvm (gas volume per unit of liquid volume per minute). For the dual phase fermentations, the aerobic fermentation proceeded for 25 h, after which aeration stopped. Subsequently, the culture was sparged with N<sub>2</sub> (1.0 vvm for 0.5 h).

### 2.3. DNA manipulations

Electrocompetent cells of *L. lactis* were made as previously described (Holo and Nes, 1989). The *L. lactis* MG1363 derivative Ace001, carrying deletions in the three lactate dehydrogenase (*ldh*, *ldhX*, *ldhB*), the phosphotransacetylase (*pta*), the alcohol dehydrogenase (*adhE*) and the butanediol dehydrogenase (*butBA*) genes is described elsewhere (Liu et al., 2016b). Ace001 was further modified by deleting the gene encoding the NADH oxidase (*noxE*). To facilitate the process we introduced a thermosensitive plasmid (pCS4564) (Liu et al., 2016a) expressing LDH from *E. coli* into *L. lactis* Ace001 to get the strain Ace002. USER<sup>TM</sup> cloning technology (Geu-Flores et al., 2007) was used to insert the upstream and downstream (800 bp) of *noxE* into the plasmid pCS1966 to make the recombinant plasmid pCS4257. The primers used are listed in Table S1. The plasmid pCS4257 was then introduced into strain Ace002, where the successful integration gave rise to erythromycin resistance. Subsequently, counter selection was carried out in the presence of 5-fluoroorotate (5-FO) to select for the resistant strain of 5-FO, where excision and loss of the

plasmid occurred (Solem et al., 2008). The resulting strain was designated Ace003 (Ace002  $\Delta noxE$ ). The strain AceN is a derivative of Ace003, which has lost the thermosensitive pCS4564 by incubation at 35°C, which is a temperature that is non-permissive for replication. The codon-optimized version of BsBdh gene (*bdhA*) (GenScript, Piscataway, USA) from *B. subtilis* was used for biosynthesis of R-BDO. The complete sequence of the synthetic gene is shown in Table S2.

#### 2.4. Quantification of metabolites and evaluation of the intracellular redox status

Cell growth was regularly monitored by measuring the optical density at 600 nm ( $OD_{600}$ ). The quantification of glucose, lactate, acetate, acetoin, R-BDO and m-BDO was carried out using a high-pressure liquid chromatography system (HPLC) equipped with a Bio-Rad Aminex HPX-87H column and a Shodex RI-101 detector (Tokyo, Japan). The mobile phase consisted of 5 mM (mmol/L)  $H_2SO_4$  and the flow rate was set at 0.5 ml/min. The column oven temperature was 60°C. To measure the NADH/NAD<sup>+</sup> ratio, samples were taken from an exponentially growing culture of *L. lactis* at an  $OD_{600}$  of 0.6, quenched using liquid nitrogen and subsequently stored at -20°C until measuring. The extraction and quantification of NADH and NAD<sup>+</sup> were performed using the NAD<sup>+</sup>/NADH-Glo<sup>TM</sup> Assay kit (Promega, Madison), following the instructions from the supplier. To evaluate the intracellular redox status, 40 ml culture samples at an  $OD_{600}$  of 1.5 were harvested by centrifugation at 6000 rpm for 10 min. Cells were re-suspended in 0.5 ml fresh SAL medium. The excitation spectrum was determined using a Plate Reader (Infinite M200 PRO), where the emission wavelength was fixed at 508 nm. For more details see Chen et al., 2011. To count the number of live cells of AceN and Ace001, we collected samples from the two cultures at the time point of 30 h and diluted to the same  $OD_{600}$  using fresh medium. Then a suitable volume of cells was plated on the surface of petri dishes, 2  $\mu$ g/ml of hemin was added for the growth of AceN.

### 3. Results and discussion

### 3.1. Inactivation of all the major NAD<sup>+</sup>-regenerating pathways in *L. lactis*

In order to investigate whether *L. lactis* is able to use respiration for efficient regeneration of NAD<sup>+</sup>, it is advantageous to eliminate the alternative ways for its regeneration. In *L. lactis*, the enzymes that are mainly responsible for regenerating NAD<sup>+</sup> include lactate dehydrogenase (LDH), alcohol dehydrogenase (AdhE), butanediol dehydrogenase (ButBA) and NADH oxidase (NoxE).

Previously, we have described a homo-acetoin producer, *L. lactis* Ace001, carrying deletions in the genes encoding LDH, PTA, AdhE and ButBA (Liu et al., 2016b). Ace001 does not grow anaerobically but can grow aerobically due to the endogenous NoxE activity. However, this strain is difficult to manipulate genetically, as the transformation efficiency is reduced, when preparing the competent cells under aerobic conditions (Liu et al., 2016a). To address this problem, we introduced a plasmid with a thermosensitive replicon expressing LDH sourced from *E. coli* (pCS4564) into Ace001, and this resulted in strain Ace002, which grew anaerobically. Subsequently we deleted the *noxE* gene and eliminated pCS4564 by aerobic incubation at 35°C in the presence of hemin. The final strain was designated AceN and the genotypes of the strains can be seen in Table 1.

In the strain AceN, almost all the competitive pathways leading away from the pyruvate node were inactivated to block formation of lactate, ethanol, acetate and formate. Formate could not be formed under the aerobic conditions used, due to oxygen inhibiting the pyruvate formate lyase (PFL) (Melchiorsen et al., 2002). Therefore, the main carbon flux was almost fully redirected towards acetoin (Fig. 1). Due to its inability to regenerate NAD<sup>+</sup>, AceN was unable to grow under anaerobic or aerobic conditions without respiration. We thus created a respiration-dependent platform strain, which could be studied further.

### 3.2. Effect of hemin concentrations on cellular performance

*L. lactis* is not able to synthesize hemin, an essential cofactor of the cytochrome oxidase (Fig. 1). Therefore, exogenous hemin needs to be added before respiration becomes active (Pedersen et al.,

2012). Before investigating the role of respiration on the cofactor balance and on chemicals production, we first tested the effect of hemin concentration on the cellular performance. Since hemin is an iron-containing protoporphyrin, high levels of hemin could be potentially toxic for the cells due to radical formation via the Fenton reaction (Kumar and Bandyopadhyay, 2005). We did not observe an inhibitory effect when the hemin levels were below 7  $\mu\text{g/ml}$ , whereas 10  $\mu\text{g/ml}$  of hemin resulted in a significantly slower growth (Fig. 2). It is well-documented that activation of respiration can result in a higher biomass yield (Duwat et al., 2001; Koebmann et al., 2008), which we also observed here. The final cell density measured by  $\text{OD}_{600}$  increased from 1.8 (0.63 gDW/L) without hemin to 2.4 (0.84 gDW/L) in the presence of hemin (Fig. 2).

Previously, we have found that the genes required for respiration, such as *noxAB*, *menABCDEFHX*, *cydABCD* and a hemin uptake operon (*fur*), were expressed constitutively regardless of the presence of hemin, and this indicates that respiration is functional once hemin is available from the environment (Liu et al., 2016a). Hemin uptake is a facilitated process, and several proteins are involved (Maresso et al., 2006). The finding, that a high hemin concentration has a negative effect on growth, is interesting, since *L. lactis* is equipped with regulatory systems to ensure hemin homeostasis, e.g., the *hrtRBA* operon encodes an efflux pump that is essential for preventing toxic intracellular build-up of hemin (Lechardeur et al., 2012).

Under normal conditions, the main function of the  $(\text{F}_1\text{F}_0)$ -ATPase in *L. lactis* is to translocate protons across the cytoplasmic membrane at the expense of ATP and to establish the essential proton gradient (Koebmann et al., 2000). It has been shown that when respiration is active, the flow of electrons through the respiratory chain also can result in proton extrusion, and thereby reduce or eliminate the drain on cellular ATP for establishing the proton gradient by the  $(\text{F}_1\text{F}_0)$ -ATPase (Blank et al., 2001). This can partly explain the higher biomass yield observed for respiring *L. lactis*. It is essential to note that for *L. lactis*, normally less than 10% of the carbon flux is used for

biomass formation (Cocaign-bousquet et al., 1996; Kleerebezem et al., 2000). However, the higher biomass achieved under respiratory conditions could be beneficial for productivity, when *L. lactis* is used as a cell factory for producing various chemicals.

### 3.3. Respiration can efficiently regenerate NAD<sup>+</sup>

We examined the growth of strain AceN in the presence of different concentrations of hemin. Fig. 3A shows that the growth of AceN was hemin-dependent as expected, and that the growth rate increased from 0.55 h<sup>-1</sup> at 0.5 µg/ml of hemin to 1.09 h<sup>-1</sup> at 2.0 µg/ml of hemin. At an initial glucose concentration of 43 mM, the final cell density reached was 4.1 (OD<sub>600</sub>) in the presence of 2 µg/ml hemin, while the OD<sub>600</sub> was only 2.3 with 1.5 µg/ml hemin and 1.7 with 1.0 µg/ml hemin.

Increasing the hemin levels further to 3 µg/ml did not result in any further changes in either the growth rate or the biomass density. We also monitored the fermentation profile of the cultures with 1.0 or 2.0 µg/ml of hemin. When the initial hemin level was set at 2.0 µg/ml, all the glucose (43 mM) could be metabolized within 8 h and 34 mM acetoin accumulated (80% of the theoretical maximum yield), while the glucose uptake and acetoin formation rates were significantly lower at 1.0 µg/ml hemin, presumably due to poorer growth (Fig. 3B). The effect of hemin on growth also correlated well with the intracellular ratio of NADH/NAD<sup>+</sup>, which decreased from 0.11 at 0.5 µg/ml of hemin to 0.05 at 2.0 µg/ml of hemin (Fig. 3C).

These data demonstrate that tuning the hemin concentration is a convenient way to regulate the intracellular NADH/NAD<sup>+</sup> ratio, which in turn affects the cellular growth and the metabolic capabilities. The growth rate of 1.0 h<sup>-1</sup>, which we achieved in the presence of hemin from 1 to 3 µg/ml, was close to that of the non-respiring wild type in the same medium. Zhu et al. (Zhu et al., 2011) previously manipulated the respiratory capacities of an engineered *E. coli* strain, which had lost the capacity for synthesizing coenzyme Q1 (the electron transfer in the respiratory system), and

found that the strain performance correlated well with Q1 supplementation. This strain could produce ethanol under aerated conditions, however, the growth rate and the biomass yield were seriously affected at low Q1 concentrations, as the main source of ATP was no longer oxidative phosphorylation. In *L. lactis* most of the ATP is generated through substrate level phosphorylation, and this allows us to design a suitable platform, where ATP generation and respiration could be uncoupled.

From our experiments, it seemed that 2  $\mu\text{g/ml}$  hemin was sufficient to fully activate respiration and drive efficient growth-coupled production of acetoin as the sole product (Fig. 3A-C). However, these experiments were carried out at relatively low cell densities due to the low glucose concentration used (43 mM). At higher cell densities, it is possible that 2  $\mu\text{g/ml}$  of hemin is insufficient to fully activate respiration in all cells, and that a higher concentration of hemin is needed. We tested this in medium containing a ten times higher initial concentration of glucose (450 mM, 81 g/L), and found that 5  $\mu\text{g/ml}$  of hemin supported the best overall growth and highest biomass accumulation ( $\text{OD}_{600}$  of 12.1). Below  $\text{OD}_{600}$  of 1.2, no difference in growth was observed when the hemin concentration was varied between 1  $\mu\text{g/ml}$  and 6  $\mu\text{g/ml}$ , however beyond this  $\text{OD}_{600}$ , an effect by the concentration of hemin became apparent (Fig. 3D).

#### **3.4. Respiration is more efficient at regenerating $\text{NAD}^+$ when compared to the NOX**

As discussed above, both respiration and NOX can regenerate  $\text{NAD}^+$ . Therefore, we decided to compare the efficiency of the fermentation process using strains Ace001 and AceN, which rely on NOX activity and respiration for growth, respectively. The fermentation was carried out using bioreactors with high initial glucose concentration (450 mM, 81 g/L), and 5  $\mu\text{g/ml}$  hemin was included in the medium for strain AceN, to fully activate respiration (see above). The final cell density ( $\text{OD}_{600}$ ) for the Ace001 culture was 9.5 and the initial glucose could not be completely

consumed within 48 h (191 mM, 34 g/L glucose left). The final titer of acetoin was 201 mM (18 g/L). In contrast, AceN grew to a higher cell density with the final OD<sub>600</sub> of 14.1. All the glucose was consumed within 48 h, leading to the formation of 371 mM (32 g/L) acetoin. The yield for acetoin production was as high as 82% of the theoretical maximum.

The improved production of acetoin in the presence of respiration on one hand could be attributed to the higher biomass, on the other hand, it has been reported that respiration can reduce oxidative stress and increase bacterial survival ability (Duwat et al., 2001). To assess whether oxidative stress could be involved, we expressed a redox-sensitive GFP (roGFP1-R12) in both Ace001 and AceN, individually. According to a study by Cannon and Remington, 2006, the green fluorescent protein variant roGFP1-R12 is a very suitable candidate for evaluating the intracellular redox status of living cells. By measuring excitation at around 400 nm and 475 nm, Chen et al., 2013 found that it was possible to evaluate the redox status of *L. lactis* grown at different temperatures. We introduced the redox sensor in the two strains, resulting in the derivatives Ace001R and AceNR, and then used the same approach to evaluate their intracellular redox status when grown under aerobic conditions. The results are shown in Table S3 (the excitation spectrum in Fig. S1). The ratio of excitation intensity was calculated to be 2.17 for Ace001R and 1.55 for AceNR, which indicated that the oxidative stress was indeed lower for the respiratory strain. In addition, the NADH/NAD<sup>+</sup> ratio and the number of viable cells (Table S4) were determined for the two cultures and it was found that the AceN culture had lower NADH/NAD<sup>+</sup> ratio and more live cells than the Ace001 culture. This indicates that the NOX activity in Ace001 is less efficient at regenerating NAD<sup>+</sup> in the selected experimental condition, and that respiration can indeed protect against oxidative stress.

### 3.5. Fine-tuning of respiration for high-yield production of R-BDO

As we demonstrated above, a fully active respiration can efficiently regenerate NAD<sup>+</sup> and allow for high yield production of acetoin from glucose. However, when producing compounds more reduced

than acetoin, it may not be desirable to have a fully active respiration, since this would deplete all of the NADH available. To test the usefulness of respiration on the production of this kind of compound, we constructed a strain able to produce the (*R,R*)-stereoisomer of butanediol (R-BDO), which can be derived from acetoin via reduction. The 2,3-butanediol dehydrogenase BsBdh from *Bacillus subtilis*, which is able to catalyze the formation of R-BDO enantiomer from acetoin (Yan et al., 2009), was introduced into strain AceN, giving rise to strain RBDO (Fig. 1). Since BsBdh consumes one of the two NADH/glucose formed in glycolysis, one NAD<sup>+</sup> has to be regenerated using respiration. To achieve fast growth as well as a high-yield production of R-BDO, we found that fine-tuning of the respiration capacity was necessary. The growth of the RBDO strain, as expected, remained hemin-dependent, but it grew significantly better than AceN under the same conditions (Fig. 5). In the presence of 1.0 µg/ml hemin, the cell density (OD<sub>600</sub>) of RBDO was 3.9 after 6.5 h, while it was only 1.7 for AceN, which demonstrated that BsBdh was able to regenerate NAD<sup>+</sup> efficiently. We found that most of the glucose was converted into R-BDO in the presence of 1.0 µg/ml of hemin and 43 mM R-BDO was synthesized from 54 mM glucose with the yield close to 80% of the theoretical maximum. In the presence of 3.0 µg/ml of hemin, large amounts of acetoin were formed (27 mM acetoin) and the yield of R-BDO dropped dramatically to 26%. These results clearly indicated that there was a need for fine-tuning the respiration capacity.

In a previous and similar study, we investigated the effect of hemin on an engineered *L. lactis* strain with the aim to produce (3*S*)-acetoin (Liu et al., 2016c), and found that only 0.2 µg/ml of hemin was optimal for growth and production, despite the same requirement for reducing power as the RBDO strain in the current study. It is likely that this difference is due to differences between the media used. For the current study, we used SA medium, whereas we used rich M17 medium previously. M17 is prepared from various rich components such as meat/beef extract, peptone and

yeast extract (Terzaghi and Sandine, 1975) that could possibly contain some hemin, which could influence growth and production.

When comparing the two possible approaches for producing R-BDO, relying on either NOX or respiration, it appears that the latter is a preferred option. In our case, for *L. lactis*, respiration is more efficient than NOX. In addition, it could be more simple to adjust the reducing power available by controlling the hemin concentration than by adjusting oxygen tension, especially for large industrial fermenters.

### 3.6. R-BDO production using a dual-phase fermentation approach

As demonstrated above, by fine-tuning respiration, it is possible to adjust the reducing power available and thus ensure high yield production of R-BDO. For producing R-BDO, it should in principle be possible to use an even more simple approach involving two stages: 1) convert a part of the glucose into acetoin using a fully active respiration, and 2) convert acetoin and glucose in a redox balanced way into R-BDO (no aeration) (Fig. 6A). We decided to test whether this was possible, and designed a two-stage fermentation process. By monitoring glucose consumption and acetoin formation, it was possible to determine the theoretical operating point (TOP) for the switch from respiration to anaerobic fermentation, as the time point when the glucose-consumption curve crossed the acetoin-formation curve. We found that it was better to switch earlier than this TOP to allow for complete consumption of the dissolved O<sub>2</sub> in the flask or fermenter. First the strategy was tested using strain RBDO in shake flasks in the presence of 3 µg/ml hemin, which was found to be sufficient to activate respiration completely (Fig. 5). After 3.5 h, we changed the fermentation mode to anaerobic, and indeed the glucose and acetoin were co-consumed. Finally 41 mM R-BDO was achieved from 54 mM glucose with a yield of 76% of the theoretical maximum (Fig. 6B). We also

optimized the operating point, and found that when glucose was in 55% molar excess of acetoin, switching was optimal to get the highest R-BDO yield (Table S5).

Recently, Liang et al., (Liang and Shen, 2017) devised a strategy where 15 g/L glucose and 5 g/L acetoin were co-fed to an *E. coli* mutant ( $\Delta ldhA \Delta adhE \Delta frd$  pKM3), which resulted in restored anaerobic growth and production of 3 g/L m-BDO. Although the co-feeding approach can allow for redox balance and decouple NADH-generation from glycolysis in the production pathway, this approach appears unfavorable due to the high cost of acetoin and is thereby incompatible with large scale production of BDO. In addition, Liang et al. found that acetoin was quite toxic for *E. coli*. In a previous study, we also observed the growth hampering effect of acetoin on *L. lactis*, and found that 20 g/L acetoin decreased the growth rate of *L. lactis* by 50% (Kandasamy et al., 2016). The switch strategy we have developed here appears to be an improvement, as it can allow for growth to a high cell density under respiratory conditions using cheap glucose, and then in the anaerobic phase, the toxic acetoin and the remaining glucose are efficiently converted into R-BDO, where fast growth is less important.

### 3.7. Efficient R-BDO production in bioreactors using the two-developed strategies

We tested the two strategies used for producing R-BDO in shake flasks using a relatively low concentration of glucose. To investigate the scalability of the approaches, we decided to test the two strategies using bioreactors and medium containing a higher glucose concentration. For strategy I, which is fine-tuning of respiration capacity, we first optimized hemin concentrations for the RBDO strain in the presence of 445 mM glucose (80 g/L). As shown in Fig. 7A, when the hemin concentration was set at 1.6  $\mu\text{g/ml}$ , we observed that R-BDO was the dominant product, and the final concentration reached 361 mM (32 g/L) in 68 h (the yield was 81% of the theoretical maximum). Minor amounts of acetoin were detectable during the fermentation process, but not after complete consumption of glucose. We also tested other hemin concentrations, 1.0  $\mu\text{g/ml}$  and 2.0

$\mu\text{g/ml}$ , and the results are shown in Fig. S2. At  $1.0 \mu\text{g/ml}$  hemin, the final cell density  $\text{OD}_{600}$  only reached 5.9, which was significantly lower than that of 9.1 achieved with  $1.6 \mu\text{g/ml}$ . At this latter hemin concentration, not all the glucose could be consumed and this resulted in a lower R-BDO titer of 217 mM (19.5 g/L) (Fig. S2A). At  $2.0 \mu\text{g/ml}$  of hemin, the final biomass density reached 10.5 ( $\text{OD}_{600}$ ) and 310 mM R-BDO (28 g/L) was produced in addition to 62 mM (5.4 g/L) of acetoin (Fig. S2B). Under the conditions applied,  $1.6 \mu\text{g/ml}$  was the most suitable level for R-BDO production.

For strategy II, which is the switch from respiration to anaerobic fermentation, we used  $5 \mu\text{g/ml}$  of hemin for the fully-active respiration and then switched to anaerobic fermentation at around 25 h by aerating with  $\text{N}_2$  for 0.5 h followed by no-aeration. We noticed that glucose and acetoin both could be consumed anaerobically, thus giving rise to R-BDO. The final concentration of R-BDO was 365 mM (33 g/L) with a yield of 82% of the theoretical maximum.

We achieved high yield production of R-BDO using both strategies and believe that each of them is suitable for producing R-BDO and other interesting chemicals, for which the amount of reducing power needs to be adjusted. Future work could include introducing the hemin biosynthetic pathway into *L. lactis*, which then could be finely controlled by using systems metabolic engineering approaches (Lee and Kim, 2015), and thereby adding hemin to the medium could be avoided.

#### 4. Conclusion

We have created an *L. lactis* platform, AceN (MG1363  $\Delta^3ldh \Delta pta \Delta adhE \Delta butBA \Delta noxE$ ), that relies on respiration in order to be able to grow, and where respiration can be fine-tuned by adjusting the hemin concentration in the growth medium. This platform appears useful for producing various useful chemicals, the biosynthesis of which require different amounts of reducing power. We used this platform for producing a high titer and high yield of acetoin by using its

maximal respiration capacity. By introducing an R-BDO forming butanediol dehydrogenase, and by adjusting the respiration capacity, we achieved efficient R-BDO production. Finally, we developed a two-stage fermentation approach for producing R-BDO. These strategies demonstrate that controlling the respiration capacity is a good way to adjust the reducing power available for chemicals production.

### Competing financial interests

The authors declare no competing financial interests.

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**Figure 1. Respiration couples the production of acetoin or R-BDO.** The cross symbol indicates competing pathways that have been inactivated. The complete respiratory chain in *L. lactis* is comprised of NADH dehydrogenase (NoxAB), menaquinone pools (mQ, mQH<sub>2</sub>) and cytochrome oxidase (Cyt bd). In order for the cytochrome oxidase to be functional, an exogenous supply of hemin is needed. Abbreviations: Als,  $\alpha$ -acetolactate synthase; AldB,  $\alpha$ -acetolactate decarboxylase; NoxE, NADH oxidase; BsBdh, butanediol dehydrogenase (BdhA) from *B. subtilis*.

**Figure 2. Effect of the hemin concentration on the cellular performance of *L. lactis* MG1363.** MG1363 was cultivated in defined SA medium with 25 mM glucose. Hemin is an iron-containing protoporphyrin and essential cofactor of cytochrome oxidase in *L. lactis*. Respiration becomes active once hemin is available from the environment.

**Figure 3. Characterization of the respiration-dependent strain AceN.** AceN (MG1363  $\Delta^3ldh \Delta pta \Delta adhE \Delta butBA \Delta noxE$ ) was cultivated in the presence of 43 mM glucose for (A)-(C) in shake flasks. (A) The growth of strain AceN is hemin-dependent. (B) Comparison of the fermentation performance of strain AceN on 1  $\mu$ g/ml (1H) or 2  $\mu$ g/ml hemin (2H). (C) The NADH/NAD<sup>+</sup> ratio as a function of hemin concentrations. (D) The effect of hemin levels on cellular growth at a high glucose concentration (450 mM glucose). Experiments were conducted in duplicates, and error bars indicate standard deviations.

**Figure 4. Efficient production of acetoin in the presence of respiration in bioreactors.** The initial glucose concentration was 450 mM (81 g/L) for both strains Ace001 and AceN. (A) The

fermentation of strain Ace001 without respiration. (B) The fermentation of strain AceN in the presence of 5  $\mu\text{g/ml}$  hemin. Experiments were conducted in duplicates and error bars indicate standard deviations.

**Figure 5. Fine-tuning respiration capacity for R-BDO biosynthesis.** The cultivation of strain RBDO (MG1363  $\Delta^3ldh \Delta pta \Delta adhE \Delta butBA \Delta noxE$  pJM002) was performed in shake flasks with 54 mM glucose. (A) Growth of RBDO was hemin-dependent. (B) High-yield production of R-BDO on 1  $\mu\text{g/ml}$  (1H). The nested figure shows the fermentation and product profile at 3  $\mu\text{g/ml}$  (3H). Experiments were conducted in duplicates and error bars indicate standard deviations.

**Figure 6. The switch from respiration to anaerobic fermentation for high-yield production of R-BDO.** (A) The stoichiometric balance of cofactors by using equal mole of glucose and acetoin under anaerobic conditions. (B) Batch fermentation of strain RBDO. The dashed line indicates the operating point for the switch from respiration to anaerobic fermentation. Phase I, fully active respiration. Shake flask fermentation was performed with the filling volume of 30 ml in 250-ml flasks at the speed of 200 rpm, and the initial glucose concentration was 54 mM. Phase II, anaerobic fermentation. The 30 ml fermentation broth was immediately transferred to 50 ml sealed tubes and was put in the static condition (slow-magnetic stirring to keep homogeneous). Experiments were conducted in duplicates and error bars indicate standard deviations.

**Figure 7. Comparison of two strategies used for producing R-BDO in bioreactors.** (A) Fine-tuning of respiration of strain RBDO with a hemin concentration of 1.6  $\mu\text{g/ml}$ . (B) The switch from fully active respiration to anaerobic fermentation. The hemin level was 5  $\mu\text{g/ml}$ . The dashed line indicated the operating point and before that, the aeration rate was 1.0 vvm of air and after that the culture was sparged with 1.0 vvm of  $\text{N}_2$  for 0.5 h and then followed by no aeration.

**Table 1. Strains and plasmids**

Designation	Genotype or description	Reference
<b><i>L. lactis</i> strains</b>		
MG1363	The wild type	Gasson, 1983
CS4363	MG1363 $\Delta^3ldh \Deltapta \Delta adhE$	Solem et al., 2013
Ace001	MG1363 $\Delta^3ldh \Deltapta \Delta adhE \Delta butBA$	Liu et al., 2016
Ace002	MG1363 $\Delta^3ldh \Deltapta \Delta adhE \Delta butBA$ pCS4564	This work
Ace003	MG1363 $\Delta^3ldh \Deltapta \Delta adhE \Delta butBA \Delta noxE$ pCS4564	This work
AceN	MG1363 $\Delta^3ldh \Deltapta \Delta adhE \Delta butBA \Delta noxE$	This work
RBDO	MG1363 $\Delta^3ldh \Deltapta \Delta adhE \Delta butBA \Delta noxE$ pJM002	This work
Ace001R	MG1363 $\Delta^3ldh \Deltapta \Delta adhE \Delta butBA$ proGFP	This work
AceNR	MG1363 $\Delta^3ldh \Deltapta \Delta adhE \Delta butBA \Delta noxE$ proGFP	This work
<b>Plasmids</b>		
pG <sup>+</sup> host8	<i>E. coli/L. lactis</i> shuttle vector, Tet <sup>R</sup> , thermosensitive replicon	Maguin et al., 1996
pCS4564	pG <sup>+</sup> host8::SP- <i>ldhA</i> ( <i>E. coli</i> )	Liu et al., 2016
pTD6	A derivative of pAK80 containing a <i>gusA</i> reporter gene	Solem et al., 2013
proGFP	For expression of roGFP-R12	Chen et al., 2013
pCS4257	For knocking out <i>noxE</i>	This work
pJM002	pTD6:: <i>bdhA</i> ( <i>Bacillus subtilis</i> )	This work

**Highlights**

- A novel *L. lactis* platform allowing for fine-tuning of the reducing power availability via respiration
- Respiration capacity modulated through hemin concentration
- High titer and yield production of acetoin by harnessing respiration
- High titer and yield production of the (*R,R*)-stereoisomer of 2,3-butanediol, either using a fine-tuned respiration or by using a dual-phase fermentation approach

