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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^+, 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.

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^+ 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^+ 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, 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

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available.

Respiration capable microorganisms often regulate the NADH/NAD^+ ratio via respiration. When NADH is oxidized into NAD^+, 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^+ and the ATP/ADP ratio, as demonstrated for *E. coli* by 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 (Koebmann et al., 2008; Tachon et al., 2010). Still most of the ATP is generated via substrate level phosphorylation even under respiratory conditions (Koebmann 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_2 (1.0 vvm for 0.5 h).

Table 1
Strains and plasmids.

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

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™ 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 Δ*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*) 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^+ ratio, samples were taken from an exponentially growing culture of *L. lactis* at an OD_{600} of 0.6, quenched