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Integrating biocompatible chemistry and manipulating cofactor partitioning in metabolically engineered *Lactococcus lactis* for fermentative production of (3S)-acetoin[†]

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Running title: Harnessing biocompatible chemistry for efficient production of (3S)-acetoin

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Abstract

Biocompatible chemistry (BC), i.e. non-enzymatic chemical reactions compatible with living organisms, is increasingly used in conjunction with metabolically engineered microorganisms for producing compounds that do not usually occur naturally. Here we report production of one such compound, (3*S*)-acetoin, a valuable precursor for chiral synthesis, using a metabolically engineered *Lactococcus lactis* strain growing under respiratory conditions with ferric iron serving as a BC component. The strain used has all competing product pathways inactivated, and an appropriate cofactor balance is achieved by fine-tuning the respiratory capacity indirectly via the hemin concentration. We achieve high-level (3*S*)-acetoin production with a final titer of 66 mM (5.8 g/L) and a high yield (71% of the theoretical maximum). To the best of our knowledge, this is the first report describing production of (3*S*)-acetoin from sugar by microbial fermentation, and the results obtained confirm the potential that lies with BC for producing useful chemicals. This article is protected by copyright. All rights reserved

Keywords

Metabolic engineering; biocompatible chemistry; (3*S*)-acetoin; cofactor partitioning

Biocompatible chemistry (BC) is emerging as a promising new field, due to the potential that lies in having non-enzymatic chemical reactions extending the chemistry of living organisms (Wallace and Balskus, 2014). In conjunction with metabolically engineered microorganisms capable of producing various compounds, the usefulness of BC becomes even more apparent. Recent examples include cyclopropanation of microbially produced styrene (Wallace and Balskus, 2015) and the production of diacetyl and (*S,S*)-2,3-butanediol (Liu et al., 2016).

Herein, we report how BC combined with metabolic engineering allows us to produce (*3S*)-acetoin, an important stereoisomer of acetoin with many applications, e.g. for synthesis of novel optically active α -hydroxyketone derivatives, pharmaceutical precursors and liquid crystal composites (Xiao and Lu, 2014). (*3R*)-acetoin, the enantiomer of (*3S*)-acetoin, can be formed readily from pyruvate by enzymatic reactions involving α -acetolactate synthase (ALS) and α -acetolactate decarboxylase (ALDB) and efficient production of this isomer has been reported previously, either using naturally occurring or engineered microorganisms (Wang et al., 2015; Zhang et al., 2014; Zhang et al., 2016). (*3S*)-acetoin is difficult to prepare using purely enzymatic transformations, but can be formed from diacetyl or *meso*-2,3-butanediol as reported by Ui et al., 1984 and Gao et al., 2013, who used non-growing cells as biocatalyst. One of the challenges of making (*3S*)-acetoin has been the low levels of its precursor - diacetyl, which is formed by non-enzymatic oxidative decarboxylation (ALOX) of α -acetolactate. α -Acetolactate is not stable and decarboxylates spontaneously into an enolate anion, which is followed by nucleophilic reactions in the presence of O₂ and then finally gives rise to diacetyl (Fig. 1). For this reason, it seems that a promising strategy for making (*3S*)-acetoin would involve metabolically engineering an efficient α -acetolactate producing cell factory that expresses a suitable (*3S*)-acetoin forming diacetyl reductase (DAR), in combination with BC for speeding up the ALOX transformation of α -acetolactate into diacetyl (Fig. 1). Another challenge is to find a solution for the cofactor

imbalance which arises, when (3*S*)-acetoin is formed from glucose and leads to a surplus of one NADH (Fig.1). All these issues could hinder the development of an efficient cell factory for high-level production by direct microbial fermentation.

Lactococcus lactis has been demonstrated to have great potential as a production organism for a broad range of interesting chemicals, due to its high glycolytic flux, well-characterized metabolic network and ease of genetic manipulation (Mazzoli et al., 2014). Normally *L. lactis* does not respire because it lacks a complete biosynthetic pathway for heme (or protoporphyrin), an essential component of the cytochrome oxidase in the electron transport chain (ETC) (Lechardeur et al., 2011), however the respiration machinery can be fully functional if heme is available from the environment, as all the other components of the ETC, such as the membrane-bound NADH dehydrogenase, menaquinone biosynthetic enzymes, and a cytochrome *bd* oxidase, are present. There are several ways in which *L. lactis* can benefit from having an active respiration, e.g. it has been reported that tolerance to oxidative stress is enhanced and that biomass formation is stimulated (Koeblmann et al., 2008). Respiration has also been demonstrated to be an excellent way to re-oxidize NADH (Liu et al., 2016).

We recently demonstrated that *L. lactis* can be converted into an efficient diacetyl producer (Liu et al., 2016). We successfully inactivated almost all alternative product pathways by deleting genes encoding lactate dehydrogenases (LDH), phosphotransacetylase (PTA), alcohol dehydrogenase (ADHE), butanediol dehydrogenases (ButBA), α -acetolactate decarboxylase (ALDB) and additionally inactivated the cytoplasmic NADH oxidase (NoxE) to optimize biomass formation. These efforts gave rise to CS4616m (MG1363 $\Delta^3ldh \Delta pta \Delta adhE \Delta butBA \Delta aldB \Delta noxE$), which was fully able to re-oxidize the surplus NADH through respiration, and most of the carbon flux (>80%) was directed to α -acetolactate/diacetyl. The α -acetolactate formed could subsequently be converted into diacetyl by adding either Fe^{3+} , Fe^{2+} or Cu^{2+} . By introducing a robust diacetyl reductase (DAR), sourced from *Enterobacter cloacae* (Li et al., 2012), into CS4616m, we obtained a strain CS4701m that readily

accumulated (2S,3S)-butanediol (SBDO) (Fig. 2A) under non-respiratory conditions (Liu et al., 2016). Since formation of SBDO from diacetyl consumes two NADH, whereas formation of (3S)-acetoin only requires one, a way to dispose of surplus NADH is needed in order to be able to produce (3S)-acetoin (Fig. 2A). One strategy that was used in *Escherichia coli* involved fine-tuning respiration by modulating the ubiquinone biosynthetic pathway (Wu et al., 2015), and this approach enabled high-yield lactate production under fully aerobic conditions. In *L. lactis*, due to the incomplete ETC caused by an incomplete hemin-biosynthesis pathway (Fig. 2A), the same effect could perhaps be achieved by adjusting the hemin concentration in the medium.

To test this we carried out preliminary growth experiments in test tubes, where CS4701m was grown in M17 medium (Terzaghi and Sandine, 1975) with 43 mM glucose, 6 mM Fe^{3+} and different hemin concentrations ranging from 0 to 5 $\mu\text{g/ml}$, and analyzed the product composition after 24 h of fermentation. Without hemin we found that SBDO was the main product as expected, and that the SBDO concentration decreased gradually with increasing hemin concentration (Table 1). Adding as little as 0.05 $\mu\text{g/ml}$ hemin had a remarkable effect on product composition, where the amount of SBDO formed was reduced by 82% (from 35 mM to 6.3 mM), while the (3S)-acetoin concentration increased from 1.1 mM to 27.3 mM. At 0.2 $\mu\text{g/ml}$ hemin, the (3S)-acetoin concentration peaked at 31.8 mM (2.8 g/L) with a yield of 0.50 C-mol/C-mol of glucose, corresponding to 74% of the theoretical maximum (Table 1). As expected, beyond this hemin concentration both (3S)-acetoin and SBDO formation were reduced, and at 5 $\mu\text{g/ml}$ hemin, the dominant product was diacetyl (25.0 mM) and only 23% of the carbon flux ended up as (3S)-acetoin (Table 1). This trend was also consistent with our observations from shake flasks (30 ml of working volume/250 ml) and we achieved a high yield of (3S)-acetoin (84%) at the same hemin level of 0.2 $\mu\text{g/ml}$ hemin (Table 1). The hemin concentration did not only affect product formation, but also the final cell density (OD_{600}), which increased from 2.1 without hemin to around 3.0 at 0.2 $\mu\text{g/ml}$ hemin, which is a 48% increase. As demonstrated by Blank et al.

(Blank et al., 2001), *L. lactis* can save some of the ATP normally used by the ATPase for generating the essential proton gradient by having an active respiration (Fig. 2A), and this additional ATP leads to an increased biomass yield which can be useful for increasing productivity, as the biomass serves as the catalyst.

These preliminary experiments clearly demonstrated that it was feasible to fine-tune the respiratory capacity of *L. lactis* for (3S)-acetoin formation by adjusting the hemin concentration (Fig. 2B). When using test tubes or shake flasks, it is difficult to ensure that sufficient oxygen is available, especially at high cell densities. In order to examine this more closely, additional experiments were carried out using bioreactors (Sartorius Biostat Q, 500 ml working volume, equipped with Clark electrode), where the dissolved oxygen (DO) level could be easily monitored. When the hemin concentration was 0.2 $\mu\text{g/ml}$, the DO level (Fig. 2C, curve I) declined slightly from 100% saturation to 78% during the first 6 h and then gradually increased to around 90%. Thus, at this hemin concentration, respiration is unlikely to alter the DO level to an extent that would affect growth or product formation. At 3 $\mu\text{g/ml}$ hemin the DO level was drastically reduced (Fig. 2C, curve III) during the first 5 h, and diacetyl was the main fermentation product demonstrating that at this hemin concentration respiration was overly active and could oxidize more of the NADH generated in glycolysis. At the intermediate hemin concentration of 0.5 $\mu\text{g/ml}$, the DO level at all times was in between that observed for 0.2 and 3 $\mu\text{g/ml}$ (Fig. 2C, curve II). Besides monitoring the DO level, the NADH/NAD⁺ ratio was also determined in order to estimate the intracellular redox state. For hemin concentrations between 0 and 1 $\mu\text{g/ml}$, a linear correlation between the NADH/NAD⁺ ratio and specific growth rate could be observed, and in this range it was possible to partition the NADH between DAR and respiration, and thereby control the strain's capacity for both cell growth and the product composition. Without hemin, the NADH/NAD⁺ ratio was high (0.08) and the strain grew relatively slowly ($\mu = 0.1 \text{ h}^{-1}$) (Fig. 2D), which indicated that the non-enzymatic ALOX flux was limiting and dictated a low SBDO production rate. At hemin concentrations

above 1 $\mu\text{g/ml}$, the opposite was observed, and a low NADH/NAD⁺ ratio (0.025) coincided with a high specific growth rate ($\mu = 0.75 \text{ h}^{-1}$). This indicates that under these conditions respiration could efficiently re-oxidize NADH, thus making it unavailable for diacetyl reduction, and therefore only small amounts of (3*S*)-acetoin accumulated (Table 1, Fig. 2). The NADH/NAD⁺ at which (3*S*)-acetoin reached the highest level was 0.065, and this corresponded to a hemin concentration of 0.2 $\mu\text{g/ml}$ (Fig. 2D).

For this reason the remaining characterization was carried out using 0.2 $\mu\text{g/ml}$ hemin. We found that within the first 12 h, nearly all the glucose (90%) had been completely consumed and the final cell density (OD₆₀₀) of 2.9 had been reached (Fig. 3A). Surprisingly (3*S*)-acetoin only constituted a small fraction of the fermentation products present (5 mM), and the remaining products were α -acetolactate (20 mM) and SBDO (10.5 mM) (Fig. 3A). Over the next 12 h the α -acetolactate and SBDO were gradually converted into (3*S*)-acetoin, which finally reached 34.5 mM (3.0 g/L) with a high yield 0.56 C-mol/C-mol of glucose (80% of the theoretical maximum). To see if it was possible to increase the titer further we doubled the glucose concentration to 93 mM, using the same hemin concentration (0.2 $\mu\text{g/ml}$), and observed the same trend as demonstrated using bioreactors (Fig. 3B). For this experiment we used shake flask conditions as a preliminary test. A (3*S*)-acetoin titer of 66 mM (5.8 g/L) was obtained, with a slightly reduced production yield (71%) when compared to the bioreactor conditions, which indicates that the hemin concentration may require further optimization when the glucose concentration is increased.

The accumulation of α -acetolactate and SBDO which we observed in the first phase, probably was caused by a limiting ALOX flux from α -acetolactate to diacetyl, despite the presence of Fe³⁺. One possibility could be to increase the Fe³⁺ concentration, however, there are drawbacks associated with this as Fe³⁺ also can have a negative effect on growth. When we increased the Fe³⁺ concentration to 10

mM, this only had a small effect on the ALOX flux (data not shown), and a better solution probably is to find a more efficient catalyst.

We did not observe any obvious formation of diacetyl during the course of fermentation, which demonstrates that the DAR was sufficiently active to allow for instantaneous conversion of the diacetyl generated into (3*S*)-acetoin and SBDO. Apparently, when the respiration capacity is limited (0.2 µg/ml hemin), the cells first convert diacetyl into SBDO to regenerate NAD⁺, which helps sustain a higher glycolytic flux and faster growth, and subsequently the accumulated α-acetolactate and SBDO are converted into (3*S*)-acetoin, probably due to respiration becoming limiting at high cell densities. To the best of our knowledge, this is the first report describing the fermentative production of (3*S*)-acetoin from glucose. This was achieved by using an approach not previously described, involving metabolic engineering, BC and co-factor partitioning by titrating the hemin concentration. The applied strategy could be very interesting for synthesis of other valuable chemicals, which are not easily obtained by conventional organic synthesis or pure enzymatic transformations.

Materials and Methods

Strains

CS4701m (MG1363 $\Delta^3ldh \Delta pta \Delta adhE \Delta butBA \Delta aldB \Delta noxE$, pJM001) is a derivative of the plasmid-free strain *Lactococcus lactis* subsp. *cremoris* MG1363 (Gasson, 1983). The plasmid pJM001, which is based on pTD6 (Solem et al., 2013), includes a synthetic codon-optimized dar (diacetyl reductase, accession no. JN035909) from *Enterobacter cloacae* with a high strength promoter (Liu et al., 2016).

Hemin optimization

The hemin optimization experiment was performed in tubes, shake flasks and subsequently, bioreactors. We used M17 medium (Oxoid, England) supplemented with 43 mM glucose and 6 mM Fe³⁺ for all the conditions. The strain CS4701m was first grown in a 25 ml tube with 5 ml of working volume and different concentrations of hemin (stock solution 0.5 mg/ml in 0.05 M NaOH) for initial optimization.

Then shake flask fermentation (250 ml conical flasks with 30 ml of working volume) was carried out. All the cultivation was carried out at 30°C and 200 rpm. In order to monitor the dissolved oxygen (DO) levels in the fermentation process and enable large scale production, the bioreactors (Sartorius Biostat Q, 500 ml working volume) were used with a constant stirring speed (150 rpm) and a constant low aeration rate around 0.02 vvm. The DO and pH were monitored during the process and samples were collected periodically for determining cell density (OD₆₀₀), glucose, α -acetolactate, diacetyl, (3S)-acetoin and SBDO concentrations. Tetracycline is kept at 5 μ g/ml for the engineered strain.

DNA techniques

The plasmid pCS1966 was used for deleting genes in *L. lactis* (Solem et al., 2008). The genes including three lactate dehydrogenase homologs (*ldh*), phosphotransacetylase (*pta*), alcohol dehydrogenase (*adhE*), butanediol dehydrogenases (*butBA*), α -acetolactate decarboxylase (*aldB*) and NADH oxidase (*noxE*) have been successfully knocked out in our previous work (Solem et al., 2013; Liu et al., 2016).

The respiration of *L. lactis* can be activated by the addition of hemin (or protoporphyrin), which is an essential cofactor of the cytochrome *bd* oxidase in the electron transport chain (Koeblmann et al., 2008).

Analytical methods

Cell growth was regularly monitored by measuring OD₆₀₀ and quantification of glucose, (3S)-acetoin and SBDO was carried out using an Ultimate 3000 high-pressure liquid chromatography system (Dionex, Sunnyvale, USA) equipped with a Aminex HPX-87H column (Bio-Rad, Hercules, USA) and a Shodex RI-101 detector (Showa Denko K.K., Tokyo, Japan). The column oven temperature was set at 60°C and the mobile phase consisted of 5 mM H₂SO₄, at a flow rate of 0.5 ml/min. Diacetyl and α -acetolactate were measured colorimetrically using a method developed by Benson et al. (Benson et al., 1996) and Westerfeld (Westerfeld, 1945). The methods for extraction of NADH and NAD⁺ were described by Liu et al., 2016 and quantification of NADH/NAD⁺ ratio were performed using the kit NAD⁺/NADH-GloTM assay (Promega).

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Figure legends

Figure 1 Combination of metabolic engineering and biocompatible chemistry for (3*S*)-acetoin production. CS4701m (MG1363 $\Delta^3ldh \Delta pta \Delta adhE \Delta butBA \Delta aldB \Delta noxE$, pJM001 (expressing DAR)) was constructed (Liu et al., 2016) and used for this purpose. The proposed mechanism for the non-enzymatic oxidative decarboxylation from α -acetolactate to diacetyl is illustrated. Abbreviation: LDH, lactate dehydrogenase; PTA, phosphotransacetylase; ADHE, alcohol dehydrogenase; ButBA, butanediol dehydrogenases; ALDB, α -acetolactate decarboxylase; ALS, α -acetolactate synthase; NoxE, NADH oxidase. DAR: diacetyl reductase from *Enterobacter cloacae*; ETC, electron transport chain; NoxAB, NADH dehydrogenases; Cyt bd, cytochrome *bd* oxidase.

Figure 2 Cofactor partitioning through the regulation of hemin concentration. (A) The 2 NADH (per glucose) formed in glycolysis can be re-oxidized in two ways, either via DAR (diacetyl reductase from *E. cloacae*) to (3*S*)-acetoin/SBDO or by the electron transport chain, where the activity of the latter can be finely controlled by hemin levels in the medium. (B) HPLC chromatograms illustrating the formation of diacetyl (R=18.9 min), (3*S*)-acetoin (R=20.1 min) and (2*S*, 3*S*)-butanediol (R=22.0 min) at different concentrations of hemin. R means retention time. (C) The dissolved oxygen (DO) levels monitored during the fermentation process under different hemin concentrations. Curve I: 0.2 $\mu\text{g/ml}$; Curve II: 0.5 $\mu\text{g/ml}$ (the drop of DO circled was caused by the addition of antifoam); Curve III: 3 $\mu\text{g/ml}$. The fermentation details were described in Materials and Methods. (D) The NADH/NAD⁺ ratio and μ (specific growth rate) as a function of hemin concentration. Experiments were conducted in duplicate and error bars indicate standard deviations.

Figure 3 Microbial fermentation for efficient production of (3*S*)-acetoin. (A) CS4701m (MG1363 Δ^3ldh Δpta $\Delta adhE$ $\Delta butBA$ $\Delta aldB$ $\Delta noxE$, pJM001 (expressing DAR)) was grown in M17 medium with 43 mM glucose, 6 mM Fe³⁺ and 0.2 μ g/ml hemin in bioreactors. Experiments were conducted in duplicate and error bars indicate standard deviations. (B) CS4701m was tested in 93 mM glucose, 6 mM Fe³⁺ and 0.2 μ g/ml hemin (Shake flasks). Experiments were conducted in duplicate and error bars indicate standard deviations.

Table 1 Optimization of hemin concentrations for strain CS4701m¹

Hemin ($\mu\text{g/ml}$)	OD ₆₀₀	Diacetyl	(2 <i>S</i> ,3 <i>S</i>)-Butanediol (mM)	(3 <i>S</i>)-Acetoin	Yield ² C-mol/C-mol of glucose	Yield ³
0 (T)	2.1	0.0	35.0	1.1	0.01	0.02
0.05 (T)	3.1	0.7	6.3	27.3	0.42	0.63
0.1 (T)	3.0	2.0	1.1	30.0	0.47	0.70
0.2 (T)	3.1	1.7	0.6	31.8	0.50	0.74
0.5 (T)	2.9	12.0	0.0	25.5	0.40	0.59
3 (T)	3.5	18.9	0.0	16.2	0.25	0.38
5 (T)	3.8	25.0	0.0	10.0	0.15	0.23
0 (F)	1.9	0.0	33.1	1.9	0.03	0.04
0.1 (F)	3.1	2.2	2.7	30.0	0.47	0.70
0.2 (F)	3.1	0.2	0.2	36.1	0.56	0.84
0.5 (F)	3.5	15.7	0.0	21.9	0.34	0.51
3 (F)	4.0	20.1	0.0	18.5	0.29	0.43
0.2 (B)	2.9	3.2	0.1	34.5	0.54	0.80
0.5 (B)	3.8	18.0	0.0	19.7	0.31	0.46
3 (B)	4.1	21.1	0.0	15.5	0.24	0.36

¹, CS4701m (MG1363 $\Delta^3ldh \Delta pta \Delta adhE \Delta butBA \Delta aldB \Delta noxE$, pJM001 (expressing DAR)) was grown in M17 medium with 43 mM glucose, 6 mM Fe³⁺ and different concentrations of hemin. The optimization was performed in Tubes (T), Shake flasks (F) and Bioreactors (B). The samples were collected after 24 h fermentation. The experiments were conducted two times, while the deviations were within $\pm 10\%$. ², yield for (3*S*)-Acetoin based on C-mol/C-mol of glucose. ³, yield for (3*S*)-Acetoin based on the theoretical maximum 0.67 C-mol/C-mol of glucose (CO₂: 0.33 C-mol/C-mol of glucose).

Figure 1

Figure 2

Figure 3