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Abstract

Lactococcus lactis subsp. *lactis* biovar diacetylactis is widely used in dairy fermentations as it can form the butter aroma compounds acetoin and diacetyl from citrate in milk. Here we explore the possibility of producing acetoin from the more abundant lactose. Starting from a dairy isolate of *L. lactis* biovar diacetylactis, we obtained a series of mutants with low lactate dehydrogenase (*ldh*) activity. One isolate, RD1M5, only had a single insertion mutation in the *ldh* gene compared to its parental strain through whole genome re-sequencing analysis. We tested the ability of RD1M5 to produce acetoin in milk. With aeration all the lactose could be consumed, and the only product was acetoin. In a simulated cheese fermentation, a 50% increase in acetoin concentration could be achieved. RD1M5 turned out to be an excellent cell factory for acetoin and was able to convert lactose in dairy waste into acetoin with high titer (41 g/L) and high yield (above 90% of the theoretical yield). Summing up, RD1M5 was found to be highly robust, and to grow excellently in milk or dairy waste. Being natural in origin opens up for applications within dairies as well as for safe production of food-grade acetoin from low-cost substrates.

Introduction

Acetoin occurs naturally in many foods, in particular in dairy products where it contributes to the buttery aroma in yoghurt, cheese, butter and butter milk, and it is commonly added to various foods to enhance flavor.^{1,2} Outside the food sector, it also has applications as a building block for synthesis of various chemicals, including diacetyl, 2-butanone and 1,3-butadiene. Due to its high value, many attempts have been made to engineer microorganisms into producing this compound, and many of them have been quite successful (Table 1). For food applications, however, recombinant microorganisms rarely find applications due to complicated regulatory demands in conjunction with public concerns regarding their use in foods.³ In EU, for instance, it is required that food additives and flavors produced by GMOs (Genetically Modified Organisms) are labeled if they constitute more than 0.9%.⁴ There are thus clear advantages of using non-genetically engineered production organisms. Such strains can be obtained in many ways, e.g. they can be isolated from different environments, or be created by spontaneous mutation or classical mutagenesis. For instance, Liu et al. isolated a *Bacillus licheniformis* strain from a solid traditional Chinese vinegar culture that could produce 41 g/L acetoin in an optimized medium.⁵ Roncal et al. obtained a mutant of the non-dairy *L. lactis* NCDO 2118 after chemical mutagenesis, which could form 59 g/L acetoin in rich two-times concentrated M17 medium.⁶ There are several reports of natural high-titer acetoin producing microorganisms (Table 1), however, all of these have drawbacks, such as lack of food approval, inability to ferment certain sugars, or they simply need a too costly substrate to support production.

Lactococcus lactis has a long record of safe use for the production of various fermented food products. Its ability to affect texture, flavor and prolong shelf-life, in combination with its generally recognized as safe status, have helped it secure an important role within the dairy industry.⁷ There are two subspecies of *L. lactis*, namely subsp. *lactis* and subsp. *cremoris*.

The subspecies were originally differentiated by phenotypic differences, which included the ability of subsp. *lactis* to consume arginine, to grow at higher temperatures or at higher salt concentrations.⁸ For subsp. *lactis*, there is one biovar that can utilize citrate, namely subsp. *lactis* biovar diacetylactis.⁹ Citrate is first transported into the cell through the citrate permease, and then cleaved by citrate lyase into acetate and oxaloacetate. Decarboxylation of oxaloacetate into pyruvate boosts the intracellular pyruvate pool sufficiently, and allows for α -acetolactate synthase (ALS) to catalyze formation of α -acetolactate. The latter compound is inherently unstable and can spontaneously be converted into diacetyl, but normally the α -acetolactate decarboxylase (ALDB) efficiently converts a large fraction into acetoin. Due to this ability to generate diacetyl and acetoin, *L. lactis* biovar diacetylactis strains are frequently included in starter cultures used for making butter, aromatic cheeses, and other fermented dairy products.

In this work, we described our efforts to generate an efficient acetoin producing *L. lactis* strain. Using chemical mutagenesis we obtained a series of variants that produced low amounts of lactate compared to their wild-type parent. One of these was characterized in detail, which included whole-genome sequencing and characterization of growth, and was shown to be an excellent acetoin producer. We demonstrated that large amounts of acetoin could be made from the lactose and citrate contained in dairy waste. This natural *L. lactis* strain of dairy origin appears to be a well-suited candidate as a production organism for acetoin, potentially for manufacturing of clean-label dairy products as acetoin production can be based on milk or even low-value dairy byproducts.

Materials and methods

Microorganisms and media

Lactococcus lactis subsp. *lactis* biovar diacetylactis SD96¹⁰ was kindly provided by Sacco S.r.l. (Cadorago, Italy). Based on strain SD96, we did adaptive laboratory evolution (ALE) in

UHT (Ultra-High-Temperature processed) milk at high temperatures, which started at 39°C and ended at 40°C. The aim of the ALE experiment was to obtain variants that were less affected by the high temperatures occurring during cheese manufacture, and that could grow rapidly in milk. A more detailed description of RD01 is not provided here, and is described elsewhere (manuscript in preparation). The strains derived from RD01, in this study, were generated by random mutagenesis with proflavine as the mutagen.

TTC (2,3,5-triphenyl tetrazolium) - containing medium was used to screen for mutants with reduced lactate dehydrogenase activity, and is composed of 5 g/L lactose, 15 g/L casein peptone, 5 g/L yeast extract, 0.5 g/L MgSO₄, 0.5 g/L ascorbic acid, 20 g/L agar, and 0.1 g/L TTC.¹¹

Mutagenesis and screening of mutants with reduced lactate dehydrogenase activity

A single colony of RD01 grown on LM17-Agar was inoculated into 5 mL of M17 with 1% lactose (LM17) in a 20-ml test tube, put in a 45° angled test tube rack, and cultivated at 30°C and 220 rpm shaking. The overnight culture was diluted with fresh LM17 medium supplemented with 10 mg/L of proflavine to a final cell density (OD₆₀₀) of 0.1. After 18 h incubation at 30°C with shaking, the cells were harvested by centrifugation (5000 ×g for 2 min) and washed three times with 0.9% NaCl. The cells were then re-suspended in fresh LM17 medium and incubated at 30°C with shaking for 1 h. After appropriate dilution in 0.9% NaCl, the cells were plated on TTC-medium to obtain single colonies. TTC can be reduced to the red compound triphenylformazan in non-acidic conditions, and colonies forming no acid, in our case lactate, appear as dark red on such plates.¹²

Whole genome re-sequencing

Genome re-sequencing of RD01 and RD1M5 was carried out by BGI (HongKong) using the Illumina Hiseq platform. The resulting data were 150-bp paired reads and were mapped to the reference genome *L. lactis* biovar diacetylactis SD96¹⁰ using Geneious Prime software

(Auckland, New Zealand). Using the same software, we detected the variants, including single nucleotide polymorphisms (SNPs), deletions and indels in RD01 and RD1M5. The *ldh* genes from all the mutant strains RD1M2 to RD1M5 were amplified with the primers *L.lac-LDH-C1F* (5'-actaataacaatgcgtatccgcactg-3') and *L.dia-LDH-C1R* (5'-cattcctacaaatggctccatgtgc-3') and then sequenced.

Fermentation in shake flasks

Milk fermentations

Pre-culturing was performed by inoculating a single colony into 5 mL of LM17 (1% lactose) in 20-mL test tubes followed by overnight growth. Cells were harvested by centrifugation at room temperature, 5000 ×g for 5 min. After removing the supernatant, cells were re-suspended in milk and inoculated into 20 mL milk in 300-mL shake flasks with desired initial cell density.

Dairy waste fermentations

Mother liquor (ML), provide by Arla food ingredients (Viby J, Denmark), is the residue obtained after recovery of lactose by crystallization from concentrated whey permeate. It is rich in lactose, amino acids and composition can vary according to the pretreatment technology used.¹³ One typical composition is shown in table S1. FCH-110 (FCH) is a by-product of Arla food ingredients' whey protein hydrolysate production and has a solid content of 11%. Its composition is shown in Table S2. ML in combination with FCH can serve as a complete fermentation medium for *L. lactis*. The seed cultures were made using ML and HCF-110. HFI-110 (HFI), free from solids, is a hydrolyzed whey protein product manufactured by Arla food ingredients. HFI can be used as a nitrogen source to support growth of *L. lactis*. The composition of HFI is shown in Table S2. The ML and HFI medium are transparent, which enables easy monitoring of bacterial growth by using a spectrophotometer (optical density at 600 nm, OD₆₀₀).

Specifically, a single colony from LM17-Agar was inoculated into 5 mL 25ML10HFI medium (25ML10HFI: 25% ML and 10% HFI) in 50-mL shake flasks and grown overnight as seed cultures. The seed-cultures were inoculated (1% v/v) into 20 mL 50ML30FCH medium (50ML30FCH: 50% ML and 30% FCH) in 300-mL shake flasks for fermentation.

Fermentation in bioreactors

The bioreactors (Sartorius Biostat A, 1L) were used with 300 mL 50ML30FCH medium. The seed-cultures were grown overnight in 25ML10HFI medium and 3 mL were used as inoculum. In all fermentations, the DO (dissolved oxygen) level was measured by Dissolved Oxygen Sensors (Mettler Toledo) and maintained above 20%, by automatic adjustment of the air flow and stirring speed. Samples were periodically collected to determine lactose, citrate, lactate and acetoin concentrations. The cell density was not measured due to the opaqueness of FCH (milky-white).

Preparation of cheese curds

The commercial starter culture C101 from New England Cheese Making SUPPLY CO. (Massachusetts, US), which contains *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*, was used to prepare cheese curd. We added 20 mg of the C101 starter into 20 liters of UHT milk and distributed the milk into 500 mL each. In group A (10 bottles), we further added RD1M5. Specifically, 20 mL overnight culture of RD1M5 ($OD_{600} = 6.5$) grown on 10ML10HFI was centrifuged and the biomass was added into 500 mL milk. In group B (another 10 bottles), 50 mL overnight culture of the wild type strain *L. lactis* biovar. *diacetylactis* SD96 ($OD_{600} = 2.6$) was centrifuged and the biomass was added into 500 mL milk. In group C (the third 10 bottles), there was no further treatment as the control. The fermentation of the three groups finished in 12 h and roughly 10 g of cheese curd was suspended and smashed in 3 mL water. After half an hour, the acetoin concentration in the liquid was measured to determine the acetoin content in the cheese curd.

Analytical methods

Quantification of lactose, lactate, acetoin, and citrate were carried out using an Ultimate high-performance liquid chromatography system equipped with a Aminex HPX-87H column (300 × 7.8 mm column) (Bio-Rad, Hercules, USA) and a Shodex RI-101 refractive index detector (Showa Denko K.K., Tokyo, Japan). The mobile phase was 5 mM H₂SO₄ with a flow rate of 0.5 mL/min and the column oven temperature was maintained at 60 °C.¹⁴ As for the detection of amino acids in mother liquor, the filtered sample was first hydrolyzed with 6 M HCl and then separated by ion exchange chromatography and detected after oxidation and derivatization with o-phthaldialdehyde.¹⁵

Results and discussion

Isolation of mutants with low lactate dehydrogenase activity

To redirect the metabolic flux towards acetoin, it is essential that the flux to lactate is reduced or eliminated (Figure 1). Starting with strain RD01, more than 50,000 colonies were screened on TTC medium to identify mutants with reduced LDH activity. In total 4 dark red colonies were isolated, and they were designated as RD1M2 to RD1M5, respectively. It was found that the *ldh* gene in all the four isolates had been mutated, and the mutations are listed in Table 2. The mutations in RD1M2 and RD1M4 resulted in single amino acid substitutions N27K and H53Y, respectively. In RD1M3, a stop codon had been introduced due to a change from C to T at position 253. In RD1M5, the region “CCGTCAAG” had been duplicated between T464 and C465, which resulted in a frameshift change.

Monnet et al.¹¹ used *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) to mutate *L. lactis* and found that most of the LDH-attenuated single mutants were unstable, as NTG mutagenesis normally results in point mutations, and most of them were G·C to A·T transitions.¹⁶ Proflavin is an acridine dye that intercalates itself between nucleic acid base pairs thereby causing basepair-deletions or basepair-duplications.¹⁷ Due to these reasons, proflavin can

induce frameshift mutations as we observed in RD1M5. We also observed point mutations in strains RD1M2 to RD1M4, which indicates that proflavin also can cause nucleotide substitutions due to its complex interactions with DNA nucleotides. Alternatively, these single point mutations could have been the outcome of natural mutation.

It is known that the inactivation of LDH can block the main metabolic flux from pyruvate to lactate and thus reduce acid production. In aerobic conditions, the NADH oxidase (NOX) can regenerate NAD^+ from NADH formed in glycolysis, which increases the pyruvate pool. Intracellular accumulation of pyruvate enables α -acetolactate synthase to convert the pyruvate into α -acetolactate, which subsequently is decarboxylated into acetoin (Figure 1). In principle pyruvate can be processed into acetyl-CoA, either by the pyruvate formate lyase (PFL) or the pyruvate dehydrogenase complex (PDHC), however, PFL is inactivated by oxygen under aerobic conditions and the PDHC requires the co-factor lipoic acid,¹⁸ which is not available in the milk.¹¹

Characterization of RD01 and its LDH defective mutants in milk fermentation

Strains obtained by using chemical mutagenesis are considered natural, and thus can be applied in dairy fermentations without any restrictions. For this reason, we decided to characterize their growth in milk. First, we investigated growth under aerated conditions, as these favor acetoin production.^{19,20} As shown in Figure 1, the main product of RD01 was lactate and this strain stopped growing and metabolizing lactose after 10 h, due to the low pH reached ($\text{pH} = 4.0$). The mutants RD1M3, RD1M4 and RD1M5 formed less lactate and large amounts of acetoin, and in 28 h the acetoin concentration reached 280 mM, at which point 145 mM of lactose and 20 mM citrate had been consumed. RD1M2 still produced some lactate, and the N27K mutation appeared not to completely abolish LDH activity, although we found it was dark red on TTC plates. This was observed for other colonies as well, that in color ranged from brown to dark red on TTC plates (data not shown). The robust growth of

the strains clearly demonstrated that mutagenesis had not affected the ability of the strains to grow in milk (i.e. ability to metabolize lactose and obtain amino acids from milk proteins).

We found that our mutant strains RD1M3 to RD1M5 were quite stable in terms of retaining ability to produce acetoin under aerobic conditions (data not shown). In *L. lactis*, the main LDH enzyme is encoded by the *ldh* gene present in the *las* (lactic acid synthesis) operon. However, there are two other genes *ldhB* and *ldhX*, that encode proteins with more than 30% amino acid identity to the *ldh*-encoded gene product.²¹ The *ldhB* gene is normally silent, but its activation has been reported in an *ldh*-mutant *L. lactis* NZ9010 upon repeated subculturing under anaerobic conditions.²² In that particular study it was noticed that the *ldh*-mutant displayed a stable phenotype when cultured aerobically, which concurs with what we find for our *ldh*-mutants in the current study (we did not investigate the stability under anaerobic conditions). Regarding *ldhX*, previous research has shown that its product has negligible LDH activity.²³

We also decided to investigate whether one of the strains, RD1M5, could enhance acetoin formation in milk, when grown statically (without active aeration). For this purpose, we prepared a blend of a commercially available starter culture, C101, with RD1M5 or its wild-type strain SD96 as a control. After curd formation due to low pH, the acetoin content of the “cheese curd” was determined. As shown in Figure S1, we observed a clear increase in acetoin content in the curd prepared using the RD1M5 containing starter (7.5 mM vs 4.9 mM using the SD96 containing starter), which demonstrates the potential of this strain for boosting butter flavor formation in dairy products.

RD1M5 whole genome re-sequencing

Due to its 8 nucleotide insertion in *ldh*, strain RD1M5 inherently is more stable than the other three *ldh* mutants obtained, which contain point mutations. For this reason we decided to focus our attention on RD1M5. To determine if other mutations, besides the one in *ldh*, had

been introduced during proflavin mutagenesis, the entire genome of RD1M5 was sequenced and compared with its parental strains RD01 and SD96.¹⁰ Genome re-sequencing revealed that the mutations in RD01, introduced in the course of the adaptive evolution of SD96, were all preserved in RD1M5 (Figure 3). These included two amino acid changes (F68L and V469L) in the CDS regions of genes encoding UDP-N-acetylmuramate-L-alanine ligase and GTP pyrophosphokinase (RelA), a large deletion (1,823,878 bp to 1,897,135 bp), one deletion in a tandem repeat ((A)₆ to (A)₅) upstream of the CodY transcription regulator and one anonymous mutation (CCC to CCT in EamA family transporter).

The strain RD01 possesses superior growth in milk at 39°C and 40°C (Table S3) when compared to its parent strain SD96. Although the reason for this behavior is not fully clear, we speculate that the mutation in UDP-N-acetylmuramate-L-alanine ligase improves the robustness of the cell wall, and enables better growth of RD01 at elevated temperatures.²⁴ The mutation in GTP pyrophosphokinase might play a role in stress response signaling as this enzyme is responsible for synthesis of the alarmone (p)ppGpp in response to amino acid starvation.²⁵ The mutation upstream of CodY is likely involved in amino acid metabolism.²⁶ A detailed investigation of the effect of the mutations, including a transcriptomic analysis of SD96 and RD01, is described elsewhere (manuscript in preparation). RD1M5 was also found to contain all the plasmids present in RD01. Consequently, compared with RD01, RD1M5 only had one additional mutation, the one in the *ldh* gene (Figure 3). Thus, the proflavin mutagenesis appeared to be very mild, which was somewhat surprising, as random mutagenesis often is considered harsh, and can lead to secondary mutations that hamper growth and overall fitness.

Acetoin from low value dairy side streams

We further explored the potential of RD1M5 as a microbial cell factory for producing acetoin from cheap feedstocks. The dairy industry generates significant volumes of low-value side

streams. One of these is whey mother liquor (ML), which is generated from whey permeate after lactose crystallization.²⁷ ML contains lactose, citrate, lactate and amino acids (Table S1). We first tested the growth of RD1M5 in 50% ML supplemented with yeast extract (YE). Without YE supplementation, the growth was quite poor. When 2% YE was added, the cell density (OD_{600}) could reach as high as 18.5, and 310 mM acetoin was generated after consumption of 160 mM lactose and 53 mM citrate (Figure 4).

ML is a leftover product of whey processing. It has been estimated that the worldwide production of cheese whey is around 1.9×10^8 ton/year, and quite large amounts of ML are available worldwide.²⁸ Dealing with these liquid wastes can be a serious challenge, as it has a high BOD (biochemical oxygen demand) and COD (chemical oxygen demand).²⁹ Recently, ML was investigated as a microbial substrate for producing ethanol and other valuable products.^{27,29} There is great potential in transforming these waste materials into useful compounds through microbial fermentation. Apparently, ML does not contain enough nitrogen source for bacterial growth and in order to make the process economically feasible, it is relevant to explore other cheaper nitrogen sources, since YE adds cost (approx. \$3.5/kg, Angel Yeast, China). We tested whey protein hydrolysate (HFI) and its side stream (FCH), which currently ends up in a biogas plant.

Assessing the potential of HFI as a nitrogen source

HFI is a hydrolyzed whey protein product and its composition is shown in Table S2. To investigate if HFI could serve a suitable nitrogen source for strain RD1M5, we carried out a series of fermentations using different concentrations of HFI (from 5% to 50%) and 50% ML as a source of lactose. This medium was termed as HFI-ML. The results showed that HFI-ML medium is able to support good growth of RD1M5 and including 10% HFI gave rise to the highest biomass yield ($OD_{600} = 7.0$) (Figure 5A). A direct correlation between biomass and acetoin production was observed.

HFI contains whey proteins and their derived peptides that can support the growth of RD1M5. *L. lactis* has an efficient proteolytic system, which includes cell-wall bound proteinase, peptide transporters and intracellular peptidases that can degrade the partially hydrolyzed protein in HFI into smaller peptides and ultimately amino acids.³⁰ We found that including more than 20% HFI in the medium resulted in hampered growth, which we believe to be due to the high salt content of HFI (Table S2).

Assessing FCH as a nitrogen source

Due to the excellent proteolytic activities of *L. lactis*, we further explored the possibility to use FCH as a nitrogen source for supporting the bacterial growth and fermentation. FCH is the by-product of the whey protein hydrolysate production, which is rich in protein/peptides and contains 11% solids. Its composition is shown in Table S2. We optimized the growth of RD1M5 on ML containing different concentrations of FCH. It was found that 30% FCH supported the highest level production of acetoin, and the 189 mM lactose was fully consumed (Figure 5B).

That a mixture of ML and FCH, two low value whey processing by-products, can be used as a fermentation substrate for producing valuable chemicals, opens up new avenues for processing dairy side streams.

Acetoin production based solely on dairy waste

Above we found that ML containing 30% FCH supported good growth and excellent acetoin production, we decided to carry out additional experiments to optimize the ML content. As shown in Figure 6, when using 40-45% ML, all the lactose could be completely consumed within 30 h, leading to the formation of 370 mM acetoin. During this process around 61 mM citrate was also consumed and very little lactate was formed. Supplementation with more ML at this point (30 h) did not increase the acetoin titer, and the lactose added remained in the broth. When 50% ML containing 225 mM lactose was used, 50 mM of lactose remained, and

the acetoin titer decreased to 350 mM. We speculated that the presence of 50% ML resulted in the bacteria being stressed osmotically, and that the extended fermentation period could lead to oxidative stress as well.

At all three concentrations of ML tested, the acetoin yield of acetoin reached more than 90% of the maximum, based on the amount of lactose and citrate consumed. The productivity was 1.1 g/L/h at 40-45% ML and 0.8 g/L/h at 50% ML (Figure 6c). Compared to other studies, we achieved the highest acetoin yield (> 0.45 g acetoin/equivalent 1 g glucose) (Table 1). The productivity was also comparably high, even though 2.0 g/L/h has been reported when using a rich and expensive medium.⁶ Considering that the fermentation substrate used in our study is based solely on a combination of waste streams, indicates that the solution has a great potential for industrial implementation.

RD1M5 appears to suffer from oxidative stress when grown in medium with high ML content

L. lactis normally is a facultative anaerobic bacterium, and the presence of O₂ can be inhibitory for its growth under some circumstances.³¹ Here we grew RD1M5 in the presence of O₂, since O₂ is required by the NADH oxidase for cofactor regeneration.³² But the presence of O₂ inevitably results in generation of ROS (reactive oxygen species) that can cause oxidative stress that in turn can damage cellular components and ultimately inhibit the fermentation performance.³³ In order to test this hypothesis, we added catalase and superoxide dismutase (SOD) into the fermentation medium. Indeed, by adding catalase (5000 U) or SOD (1000 U), it was possible to improve acetoin production significantly. When SOD was added, the acetoin titer reached 483 mM after 48 h of fermentation, whereas acetoin production stopped after 44 h without SOD added (Figure 7A). These results demonstrate that scavenging of ROS is beneficial for maintaining the metabolic activities of RD1M5. In the

presence of catalase or SOD, all the lactose in 50% ML could be completely consumed and converted into acetoin.

L. lactis does not possess a catalase or other antioxidant metalloenzymes that can degrade toxic H_2O_2 into H_2O molecules and O_2 .^{34,35} It has been reported that the presence of catalase can help improve growth of *L. lactis* under oxidative stress conditions.³⁶ To cope with oxidative stress, *L. lactis* has inducible SOD activities, which are dependent on carbon source and growth conditions,^{37,38} as well as NADH oxidase activities. RecA (the DNA recombination/repair protein) has been demonstrated to be involved in the oxidative stress response, and a *recA* mutant is highly sensitive to aeration.³⁹ Even though *L. lactis* has intrinsic capacities to deal with oxidative stress, we found that adding either catalase or SOD in the medium was beneficial for aerated fermentation.

In addition to these enzymatic chemistry against oxidative stress, Mn^{2+} has been found to serve as a non-enzymatic SOD for ROS clearance,^{40,41} and we decided to test if Mn^{2+} could have a beneficial effect in our fermentations. Different concentrations of Mn^{2+} were tested, and it was shown to be extremely beneficial for stimulating acetoin production, and indeed serve the same role as SOD. In the presence of 0.2 mM Mn^{2+} , we could achieve 425 mM of acetoin in 56 h and the titer could be increased to 452 mM when using 1 or 2 mM of Mn^{2+} (Figure 7A). A similar Mn^{2+} -stimulation was also documented for *Lactobacillus casei* to produce lactic acid from whey permeate.⁴² The chemical mechanism by which Mn^{2+} can detoxify ROS still remains largely unknown. *In vitro* experimental studies by Barnese et al. confirmed that manganese phosphate and manganese carbonate under physiologically relevant conditions can catalyse superoxide disproportionation.⁴³

Scaling-up acetoin production in bioreactors

Because of the beneficial effect of Mn^{2+} , we added 1 mM Mn^{2+} into the 50% ML and 30% FCH medium, and carried out fermentations in bioreactors (Sartorius Biostat A, 1L). As

shown in Figure 7B, all the lactose was completely consumed in 56 h and the acetoin concentration reached 456 mM (41 g/L) with a high yield of 93%. There was still 87 mM of unmetabolized citrate in the medium, and only half of the citrate was consumed. During the entire fermentation process the DO levels were maintained above 20% of air saturation, since we found that O₂ was essential for cofactor regeneration and for avoiding the formation of 2,3-butanediol (Figure S2).

We found that only half the citrate could be metabolized, and one reason for this could be due to the increase in pH that accompanies citrate metabolization. The initial pH of the ML/HCF medium was around 5.5, and this pH has been reported to be optimal for citrate to be metabolized,⁴⁴ as expression of the citrate permease is induced by low pH and transport of divalent citrate is most efficient around pH 5.5.⁴⁵ As citrate is metabolized, pH increases and finally citrate uptake is halted, that is why citrate was mainly metabolized in the first 25 h (Figure 7B). During the fermentation, we maintained the DO levels high, as oxygen is needed for cofactor regeneration, i.e. NADH reoxidation by the NADH oxidase. We have previously found that insufficient O₂ levels could promote formation of 2,3-butanediol and the outcome was a lower acetoin titer.³² Controlling the O₂ level has also been found to be important for other microorganism to produce acetoin.²⁰

In conclusion, we have generated an efficient acetoin forming *L. lactis* strain without using genetic tools, and have shown that it can be used both in dairy fermentations for contributing butter aroma, and as an excellent cell factory for producing acetoin from lactose containing dairy waste. The acetoin forming strain RD1M5, is unique as it forms acetoin as a major fermentation product, and it grows well in both milk and in a medium consisting of only dairy waste.

Supporting Information

The composition of mother liquor; the composition of FCH and HFI; acidification of UHT milk at high temperature, measured by the time until pH 5.2 was reached; the acetoin content in cheese curd; fermentation without O₂ control.

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Figure 1. The metabolic pathway for synthesis of acetoin. Abbreviations: ALS, α -acetolactate synthase. ALDB, α -acetolactate decarboxylase. ACK, acetate kinase. ADH, alcohol dehydrogenase. LDH, lactate dehydrogenase. NOX, NADH oxidase. PTA, phosphoacetyltransferase. PDHC, pyruvate dehydrogenase complex. PFL, pyruvate formate lyase.

Figure 2. Characterization of RD01 and derived low-acid forming mutants in aerated milk. A. Lactose consumption. B. Acetoin production. C. Citrate consumption. and D. Lactate production. All the experiments were carried out two times independently.

Figure 3. The genome information of SD96 and its derivatives RD01 and RD1M5. RD01 is adaptively evolved at high temperatures based on the industrial dairy strain SD96. RD1M5 is one mutant strain of RD01 treated with proflavin.

Figure 4. Effect of yeast extract (YE) on the performance of RD1M5 using 50% ML. The initial OD₆₀₀ was 0.25. All the fermentations were carried out in 20 mL medium using 300-mL shake flasks for two times independently. Error bars indicate standard deviations.

Figure 5. Effect of HFI and FCH on the performance of RD1M5 in 50% ML. Initial lactose was 189 mM. The samples were taken in 24 h. The samples were taken in 42 h. All the fermentations were carried out in 20 mL medium using 300-mL shake flasks for two times independently. Error bars indicate standard deviations.

Figure 6. Fermentation of the strain RD1M5 in the medium ML containing FCH. All the fermentations were carried out in 20 mL medium using 300-mL shake flasks for two times independently. Error bars indicate standard deviations. At 28 h, 4 mL of 100% ML were added into the shakers with 40% and 45% ML. (A) Lactose concentrations; (B) Acetoin production; (C) The yield and productivity of acetoin during the fermentation of RD1M5 in dairy waste. 40 (45, 50) ML30FCH: 40 (45, 50) % ML + 30% FCH.

Figure 7. Fermentation of the strain RD1M5 in 50% ML (225 mM lactose) with 30% FCH.

A: All the fermentations were carried out in 20 mL medium using 300-mL shake flasks for two times independently. SOD, superoxide dismutase. B: The fermentation was carried out in 300 mL medium in bioreactors (Sartorius Biostat A, 1L). The experiments were carried out two times independently. Error bars indicate standard deviations.

Table 1. Acetoin production in microorganisms.

	Microorganism	Approach ^a	Titer and yield	Substrate	Reference
GMO approach	<i>Saccharomyces cerevisiae</i>	Deletion: ADH, GPD, BDH Introduction: ALS, ALDB, NOX	100 g/L 0.44 g/g	YPD medium	46
	<i>Bacillus subtilis</i>	Deletion: BDH Overexpression: NOX	57 g/L 0.32 g/g	Complex medium containing BE	47
	<i>Lactococcus lactis</i>	Deletion: LDH, PTA, ADH, BDH	32 g/L 0.40 g/g	Synthetic medium with YE	32
	<i>Enterobacter cloacae</i>	Deletion: LDH, BDH, ADH, FR. Overexpression: GP, NOX	46 g/L 0.28 g/g	Lignocellulosic hydrolysate	48
Non-GMO approach	<i>Bacillus licheniformis</i>	Isolate from solid cultures of traditional Chinese vinegar	41 g/L 0.40 g/g	Complex medium containing YE, BE	5
	<i>B. subtilis</i>	Isolate from samples of sea sediment	61 g/L 0.42 g/g	Molasses with YE	49
	<i>L. lactis</i>	Chemical mutagenesis using ethyl methanesulfonate	59 g/L 0.35 g/g	Concentrated M17	6
	<i>L. lactis</i>	Chemical mutagenesis using proflavine	41 g/L 0.45 g/g	Complete dairy waste stream	this work

^aAbbreviations: GMO, genetically modified organism. ADH, alcohol dehydrogenase. GPD, glycerol-3-phosphate dehydrogenase. BDH, butanediol dehydrogenase. ALS, α -acetolactate synthase. ALDB, α -acetolactate decarboxylase. NOX, NADH oxidase. LDH, lactate dehydrogenase. PTA, phosphoacetyltransferase. FR, fumarate reductase. GP, galactose permease. YE, yeast extract. BE, beef extract.

Table 2. The mutations contained in their *ldh* genes of RD01-derived mutant strains.

Mutant	Mutation in the <i>ldh</i> gene
RD1M2	C81G(N27K)
RD1M3	C253T(Q85Stop)
RD1M4	C157T(H53Y)
RD1M5	insertion CCGTCAAG between T464 and C465 causing frameshift mutation

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Figure 6.

Figure 7.

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