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Published in:
Journal of Agricultural and Food Chemistry

Link to article, DOI:
10.1021/acs.jafc.0c00882

Publication date:
2020

Document Version
Peer reviewed version

Link back to DTU Orbit

Citation (APA):
Liu, J., Chen, L., Dorau, R., Lillevang, S. K., Jensen, P. R., & Solem, P. C. (2020). From waste to taste - Efficient production of the butter aroma compound acetoin from low value dairy side streams using a natural (non-engineered) Lactococcus lactis dairy isolate. *Journal of Agricultural and Food Chemistry*, *68*(21), 5891–5899. https://doi.org/10.1021/acs.jafc.0c00882

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JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Biotechnology and Biological Transformations

From waste to taste – Efficient production of the butter aroma compound acetoin from low value dairy side streams using a natural (non-engineered) Lactococcus lactis dairy isolate

Jianming Liu, Lin Chen, Robin Dorau, Søren Kristian Lillevang, Peter Ruhdal Jensen, and Prof. Christian Solem

J. Agric. Food Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jafc.0c00882 • Publication Date (Web): 04 May 2020

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From waste to taste – Efficient production of the butter aroma compound acetoin from low value dairy side streams using a natural (non-engineered) *Lactococcus lactis* dairy isolate

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22

23 **Abstract**

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

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46 **Introduction**

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

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

71 The subspecies were orginally differentiated by phenotypic differences, which included the
72 ability of subsp. *lactis* to consume arginine, to grow at higher temperatures or at higher salt
73 concentrations.[8] For subsp. *lactis*, there is one biovar that can utilize citrate, namely subsp.
74 *lactis* biovar diacetylactis.[9] Citrate is first transported into the cell through the citrate
75 permease, and then cleaved by citrate lyase into acetate and oxaloacetate. Decarboxylation of
76 oxaloacetate into pyruvate boosts the the intracellular pyruvate pool sufficiently, and allows
77 for α-acetolactate synthase (ALS) to catalyze formation of α-acetolactate. The latter
78 compound is inherently unstable and can spontanously be converted into diacetyl, but
79 normally the α-acetolactate decarboxylase (ALDB) efficiently converts a large fraction into
80 acetoin. Due to this ability to generate diacetyl and acetoin, *L. lactis* biovar diacetylactis
81 strains are frequently included in starter cultures used for making butter, aromatic cheeses,
82 and other fermented dairy products.
83 In this work, we described our efforts to generate an efficient acetoin producing *L. lactis*
84 strain. Using chemical mutagenesis we obtained a series of variants that produced low
85 amounts of lactate compared to their wild-type parent. One of these was characterized in
86 detail, which included whole-genome sequencing and characterization of growth, and was
87 shown to be an excellent acetoin producer. We demonstrated that large amounts of acetoin
88 could be made from the lactose and citrate contained in dairy waste. This natural *L. lactis*
89 strain of dairy origin appears to be a well-suited candidate as a production organism for
90 acetoin, potentially for manufacturing of clean-label dairy products as acetoin production can
91 be based on milk or even low-value dairy byproducts.

92 Materials and methods

93 Microorganisms and media

94 *Lactococcus lactis* subsp. *lactis* biovar diacetylactis SD96 [10] was kindly provide by Sacco
95 S.r.l. (Cadorago, Italy). Based on strain SD96, we did adaptive laboratory evolution (ALE) in

96 UHT (Ultra-High-Temperature processed) milk at high temperatures, which started at 39°C
97 and ended at 40°C. The aim of the ALE experiment was to obtain variants that were less
98 affected by the high temperatures occurring during cheese manufacture, and that could grow
99 rapidly in milk. A more detailed description of RD01 is not provided here, and is described
100 elsewhere (manuscript in preparation). The strains derived from RD01, in this study, were
101 generated by random mutagenesis with proflavine as the mutagen.
102 TTC (2,3,5-triphenyl tetrazolium) - containing medium was used to screen for mutants with
103 reduced lactate dehydrogenase activity, and is composed of 5 g/L lactose, 15 g/L casein
104 peptone, 5 g/L yeast extract, 0.5 g/L $MgSO_4$, 0.5 g/L ascorbic acid, 20 g/L agar, and 0.1 g/L
105 TTC.[11]

106 **Mutagenesis and screening of mutants with reduced lactate dehydrogenase activity**

107 A single colony of RD01 grown on LM17-Agar was inoculated into 5 mL of M17 with 1%
108 lactose (LM17) in a 20-ml test tube, put in a 45° angled test tube rack, and cultivated at 30°C
109 and 220 rpm shaking. The overnight culture was diluted with fresh LM17 medium
110 supplemented with 10 mg/L of proflavine to a final cell density (OD_{600}) of 0.1. After 18 h
111 incubation at 30°C with shaking, the cells were harvested by centrifugation (5000 ×g for 2
112 min) and washed three times with 0.9% NaCl. The cells were then re-suspended in fresh
113 LM17 medium and incubated at 30°C with shaking for 1 h. After appropriate dilution in 0.9%
114 NaCl, the cells were plated on TTC-medium to obtain single colonies. TTC can be reduced to
115 the red compound triphenylformazan in non-acidic conditions, and colonies forming no acid,
116 in our case lactate, appear as dark red on such plates.[12]

117 **Whole genome re-sequencing**

118 Genome re-sequencing of RD01 and RD1M5 was carried out by BGI (HongKong) using the
119 Illumina Hiseq platform. The resulting data were 150-bp paired reads and were mapped to the
120 reference genome *L. lactis* biovar diacetylactis SD96 [10] using Geneious Prime software

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

126 **Fermentation in shake flasks**

127 Milk fermentations

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

133 Dairy waste fermentations

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

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

150 **Fermentation in bioreactors**

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

158 **Preparation of cheese curds**

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

171 **Analytical methods**

172 Quantification of lactose, lactate, acetoin, and citrate were carried out using an Ultimate high-
173 performance liquid chromatography system equipped with a Aminex HPX-87H column (300
174 × 7.8 mm column) (Bio-Rad, Hercules, USA) and a Shodex RI-101 refractive index detector
175 (Showa Denko K.K., Tokyo, Japan). The mobile phase was 5 mM H_2SO_4 with a flow rate of
176 0.5 mL/min and the column oven temperature was maintained at 60 ^oC.[14] As for the detection
177 of amino acids in mother liquor, the filtered sample was first hydrolyzed with 6 M HCl and
178 then separated by ion exchange chromatography and detected after oxidation and
179 derivatization with o-phthaldialdehyde.[15]

180 **Results and discussion**

181 **Isolation of mutants with low lactate dehydrogenase activity**

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

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

196 induce frameshift mutations as we observed in RD1M5. We also observed point mutations in
197 strains RD1M2 to RD1M4, which indicates that proflavin also can cause nucleotide
198 substitutions due to its complex interactions with DNA nucleotides. Alternatively, these
199 single point mutations could have been the outcome of natural mutation.
200 It is known that the inactivation of LDH can block the main metabolic flux from pyruvate to
201 lactate and thus reduce acid production. In aerobic conditions, the NADH oxidase (NOX) can
202 regenerate NAD^+ from NADH formed in glycolysis, which increases the pyruvate pool.
203 Intracellular accumulation of pyruvate enables α-acetolactate synthase to convert the
204 pyruvate into α-acetolactate, which subsequently is decarboxylated into acetoin (Figure 1). In
205 principle pyruvate can be processed into acetyl-CoA, either by the pyruvate formate lyase
206 (PFL) or the pyruvate dehydrogenase complex (PDHC), however, PFL is inactivated by
207 oxygen under aerobic conditions and the PDHC requires the co-factor lipoic acid,[18] which is
208 not available in the milk.[11]

209 **Characterization of RD01 and its LDH defective mutants in milk fermentation**

210 Strains obtained by using chemical mutagenesis are considered natural, and thus can be
211 applied in dairy fermentations without any restrictions. For this reason, we decided to
212 characterize their growth in milk. First, we investigated growth under aerated conditions, as
213 these favor acetoin production.[19,20] As shown in Figure 1, the main product of RD01 was
214 lactate and this strain stopped growing and metabolizing lactose after 10 h, due to the low pH
215 reached (pH = 4.0). The mutants RD1M3, RD1M4 and RD1M5 formed less lactate and large
216 amounts of acetoin, and in 28 h the acetoin concentration reached 280 mM, at which point
217 145 mM of lactose and 20 mM citrate had been consumed. RD1M2 still produced some
218 lactate, and the N27K mutation appeared not to completely abolish LDH activity, although
219 we found it was dark red on TTC plates. This was observed for other colonies as well, that in
220 color ranged from brown to dark red on TTC plates (data not shown). The robust growth of

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

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

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

242 **RD1M5 whole genome re-sequencing**

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

246 been introduced during proflavin mutagenesis, the entire genome of RD1M5 was sequenced
247 and compared with its parental strains RD01 and SD96.[10] Genome re-sequencing revealed
248 that the mutations in RD01, introduced in the course of the adaptive evolution of SD96, were
249 all preserved in RD1M5 (Figure 3). These included two amino acid changes (F68L and
250 V469L) in the CDS regions of genes encoding UDP-N-acetylmuramate-L-alanine ligase and
251 GTP pyrophosphokinase (RelA), a large deletion (1,823,878 bp to 1,897,135 bp), one
252 deletion in a tandem repeat ((A)6 to (A)5) upstream of the CodY transcription regulator and
253 one anonymous mutation (CCC to CCT in EamA family transporter).
254 The strain RD01 possesses superior growth in milk at 39°C and 40°C (Table S3) when
255 compared to its parent strain SD96. Although the reason for this behavior is not fully clear,
256 we speculate that the mutation in UDP-N-acetylmuramate-L-alanine ligase improves the
257 robustness of the cell wall , and enables better growth of RD01 at elevated temperatures.[24]
258 The mutation in GTP pyrophosphokinase might play a role in stress response signaling as this
259 enzyme is responsible for synthesis of the alarmone (p)ppGpp in response to amino acid
260 starvation.[25] The mutation upstream of CodY is likely involved in amino acid metabolism.[26]
261 A detailed investigation of the effect of the mutations, including a transcriptomic analysis of
262 SD96 and RD01, is described elsewhere (manuscript in preparation). RD1M5 was also found
263 to contain all the plasmids present in RD01. Consequently, compared with RD01, RD1M5
264 only had one additional mutation, the one in the *ldh* gene (Figure 3). Thus, the proflavin
265 mutagenesis appeared to be very mild, which was somewhat surprising, as random
266 mutagenesis often is considered harsh, and can lead to secondary mutations that hamper
267 growth and overall fitness.
268 **Acetoin from low value dairy side streams**
269 We further explored the potential of RD1M5 as a microbial cell factory for producing acetoin
270 from cheap feedstocks. The dairy industry generates significant volumes of low-value side

271 streams. One of these is whey mother liquor (ML), which is generated from whey permeate
272 after lactose crystallization.[27] ML contains lactose, citrate, lactate and amino acids (Table
273 S1). We first tested the growth of RD1M5 in 50% ML supplemented with yeast extract (YE).
274 Without YE supplementation, the growth was quite poor. When 2% YE was added, the cell
275 density (OD_{600}) could reach as high as 18.5, and 310 mM acetoin was generated after
276 consumption of 160 mM lactose and 53 mM citrate (Figure 4).
277 ML is a leftover product of whey processing. It has been estimated that the worldwide
278 production of cheese whey is around 1.9×10^8 ton/year, and quite large amounts of ML are
279 available worldwide.[28] Dealing with these liquid wastes can be a serious challenge, as it has
280 a high BOD (biochemical oxygen demand) and COD (chemical oxygen demand).[29] Recently,
281 ML was investigated as a microbial substrate for producing ethanol and other valuable
282 products.[27,29] There is great potential in transforming these waste materials into useful
283 compounds through microbial fermentation. Apparently, ML does not contain enough
284 nitrogen source for bacterial growth and in order to make the process economically feasible,
285 it is relevant to explore other cheaper nitrogen sources, since YE adds cost (approx. $3.5/kg,
286 Angel Yeast, China). We tested whey protein hydrolysate (HFI) and its side stream (FCH),
287 which currently ends up in a biogas plant.
288 **Assessing the potential of HFI as a nitrogen source**
289 HFI is a hydrolyzed whey protein product and its composition is shown in Table S2. To
290 investigate if HFI could serve a suitable nitrogen source for strain RD1M5, we carried out a
291 series of fermentations using different concentrations of HFI (from 5% to 50%) and 50% ML
292 as a source of lactose. This medium was termed as HFI-ML. The results showed that HFI-ML
293 medium is able to support good growth of RD1M5 and including 10% HFI gave rise to the
294 highest biomass yield ($OD_{600} = 7.0$) (Figure 5A). A direct correlation between biomass and
295 acetoin production was observed.

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

302 **Assessing FCH as a nitrogen source**

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

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

313 **Acetoin production based solely on dairy waste**

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

321 the acetoin titer decreased to 350 mM. We speculated that the presence of 50% ML resulted
322 in the bacteria being stressed osmotically, and that the extended fermentation period could
323 lead to oxidative stress as well.
324 At all three concentrations of ML tested, the acetoin yield of acetoin reached more than 90%
325 of the maximum, based on the amount of lactose and citrate consumed. The productivity was
326 1.1 g/L/h at 40-45% ML and 0.8 g/L/h at 50% ML (Figure 6c). Compared to other studies,
327 we achieved the highest acetoin yield (> 0.45 g acetoin/equivalent 1 g glucose) (Table 1). The
328 productivity was also comparably high, even though 2.0 g/L/h has been reported when using
329 a rich and expensive medium.[6] Considering that the fermentation substrate used in our study
330 is based solely on a combination of waste streams, indicates that the solution has a great
331 potential for industrial implementation.

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

334 *L. lactis* normally is a facultative anaerobic bacterium, and the presence of O_2 can be
335 inhibitory for its growth under some circumstances.[31] Here we grew RD1M5 in the presence
336 of O_2, since O_2 is required by the NADH oxidase for cofactor regeneration.[32] But the
337 presence of O_2 inevitably results in generation of ROS (reactive oxygen species) that can
338 cause oxidative stress that in turn can damage cellular components and ultimately inhibit the
339 fermentation performance.[33] In order to test this hypothesis, we added catalase and
340 superoxide dismutase (SOD) into the fermentation medium. Indeed, by adding catalase (5000
341 U) or SOD (1000 U), it was possible to improve acetoin production significantly. When SOD
342 was added, the acetoin titer reached 483 mM after 48 h of fermentation, whereas acetoin
343 production stopped after 44 h without SOD added (Figure 7A). These results demonstrate that
344 scavenging of ROS is beneficial for maintaining the metabolic activities of RD1M5. In the

345 presence of catalase or SOD, all the lactose in 50% ML could be completely consumed and
346 converted into acetoin.
347 *L. lactis* does not possess a catalase or other antioxidant metalloenzymes that can degrade
348 toxic H_2O_2 into H_2O molecules and O_2.[34,35] It has been reported that the presence of catalase
349 can help improve growth of *L. lactis* under oxidative stress conditions.[36] To cope with
350 oxidative stress, *L. lactis* has inducible SOD activities, which are dependent on carbon
351 source and growth conditions,[37,38] as well as NADH oxidase activities. RecA (the DNA
352 recombination/repair protein) has been demonstrated to be involved in the oxidative stress
353 response, and a *recA* mutant is highly sensitive to aeration.[39] Even though *L. lactis* has
354 intrinsic capacities to deal with oxidative stress, we found that adding either catalase or SOD
355 in the medium was beneficial for aerated fermentation.
356 In addition to these enzymatic chemistry against oxidative stress, Mn^{2+} has been found to
357 serve as a non-enzymatic SOD for ROS clearance,[40,41] and we decided to test if Mn^{2+} could
358 have a beneficial effect in our fermentations. Different concentrations of Mn^{2+} were tested,
359 and it was shown to be extremely beneficial for stimulating acetoin production, and indeed
360 serve the same role as SOD. In the presence of 0.2 mM Mn^{2+}, we could achieve 425 mM of
361 acetoin in 56 h and the titer could be increased to 452 mM when using 1 or 2 mM of Mn^{2+}
362 (Figure 7A). A similar Mn^{2+}-stimulation was also documented for *Lactobacillus casei* to
363 produce lactic acid from whey permeate.[42] The chemical mechanism by which Mn^{2+} can
364 detoxify ROS still remains largely unknown. *In vitro* experimental studies by Barnese et al.
365 confirmed that manganese phosphate and manganese carbonate under physiologically
366 relevant conditions can catalyse superoxide disproportionation.[43]

367 **Scaling-up acetoin production in bioreactors**

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

370 shown in Figure 7B, all the lactose was completely consumed in 56 h and the acetoin
371 concentration reached 456 mM (41 g/L) with a high yield of 93%. There was still 87 mM of
372 unmetabolized citrate in the medium, and only half of the citrate was consumed. During the
373 entire fermentation process the DO levels were maintained above 20% of air saturation, since
374 we found that O_2 was essential for cofactor regeneration and for avoiding the formation of
375 2,3-butanediol (Figure S2).
376 We found that only half the citrate could be metabolized, and one reason for this could be due
377 to the increase in pH that accompanies citrate metabolization. The initial pH of the ML/HCF
378 medium was around 5.5, and this pH has been reported to be optimal for citrate to be
379 metabolized,[44] as expression of the citrate permease is induced by low pH and transport of
380 divalent citrate is most efficient around pH 5.5.[45] As citrate is metabolized, pH increases and
381 finally citrate uptake is halted, that is why citrate was mainly metabolized in the first 25 h
382 (Figure 7B). During the fermentation, we maintained the DO levels high, as oxygen is needed
383 for cofactor regeneration, i.e. NADH reoxidation by the NADH oxidase. We have previously
384 found that insufficient O_2 levels could promote formation of 2,3-butanediol and the outcome
385 was a lower acetoin titer.[32] Controlling the O_2 level has also been found to be important for
386 other microorganism to produce acetoin.[20]
387 In conclusion, we have generated an efficient acetoin forming *L. lactis* strain without using
388 genetic tools, and have shown that it can be used both in dairy fermentations for contributing
389 butter aroma, and as an excellent cell factory for producing acetoin from lactose containing
390 dairy waste. The acetoin forming strain RD1M5, is unique as it forms acetoin as a major
391 fermentation product, and it grows well in both milk and in a medium consisting of only
392 dairy waste.

393 **Supporting Information**

394 The composition of mother liquor; the composition of FCH and HFI; acidification of UHT
395 milk at high temperature, measured by the time until pH 5.2 was reached; the acetoin content
396 in cheese curd; fermentation without O_2 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 OD_{600} 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	*Saccharomyces cerevisiae*	**Deletion**: ADH, GPD, BDH **Introduction**: ALS, ALDB, NOX	100 g/L 0.44 g/g	YPD medium	46
	Bacillus subtilis	**Deletion**: BDH **Overexpression**: NOX	57 g/L 0.32 g/g	Complex medium containing BE	47
	Lactococcus lactis	**Deletion**: LDH, PTA, ADH, BDH	32 g/L 0.40 g/g	Synthetic medium with YE	32
	Enterobacter cloacae	**Deletion**: LDH, BDH, ADH, FR. **Overexpression**: GP, NOX	46 g/L 0.28 g/g	Lignocellulosic hydrolysate	48
Non-GMO approach	*Bacillus licheniformis*	Isolate from solid cultures of traditional Chinese vinegar	41 g/L 0.40 g/g	Complex medium containing YE, BE	5
	B. subtilis	Isolate from samples of sea sediment	61 g/L 0.42 g/g	Molasses with YE	49
	L. lactis	Chemical mutagenesis using ethyl methanesulfonate	59 g/L 0.35 g/g	Concentrated M17	6
	L. lactis	Chemical mutagenesis using proflavine	41 g/L 0.45 g/g	**Complete dairy waste stream**	this work

[a]Abbreviations: 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 *ldh* gene**
RD1M2	C81G(N27K)
RD1M3	C253T(Q85Stop)
RD1M4	C157T(H53Y)
RD1M5	insertion CCGTCAAG between T464 and C465 causing frameshift mutation

Figure 1.

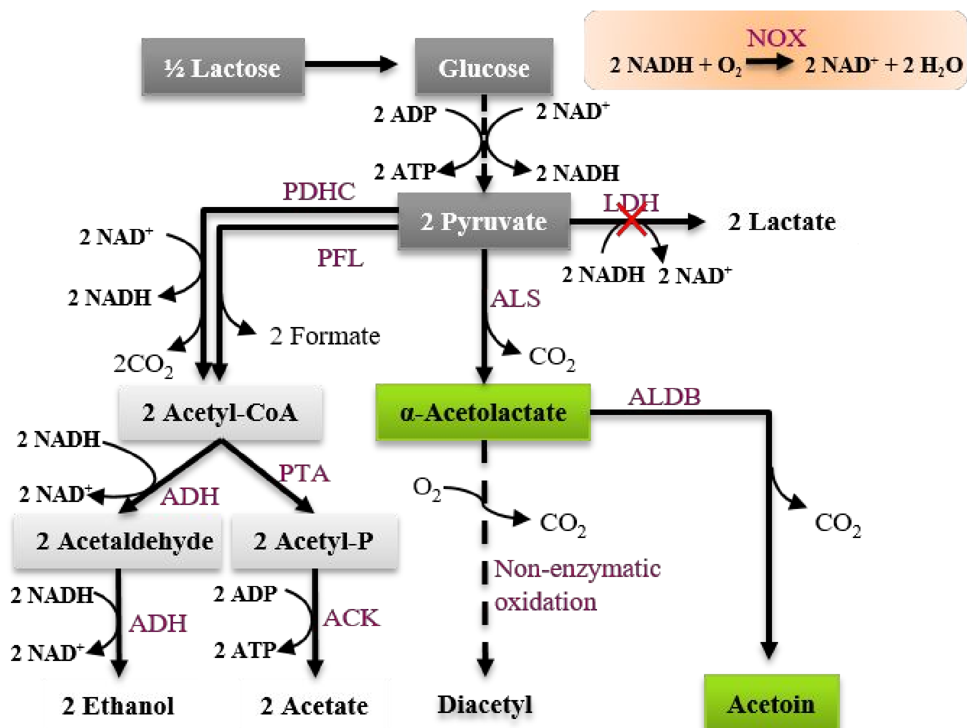
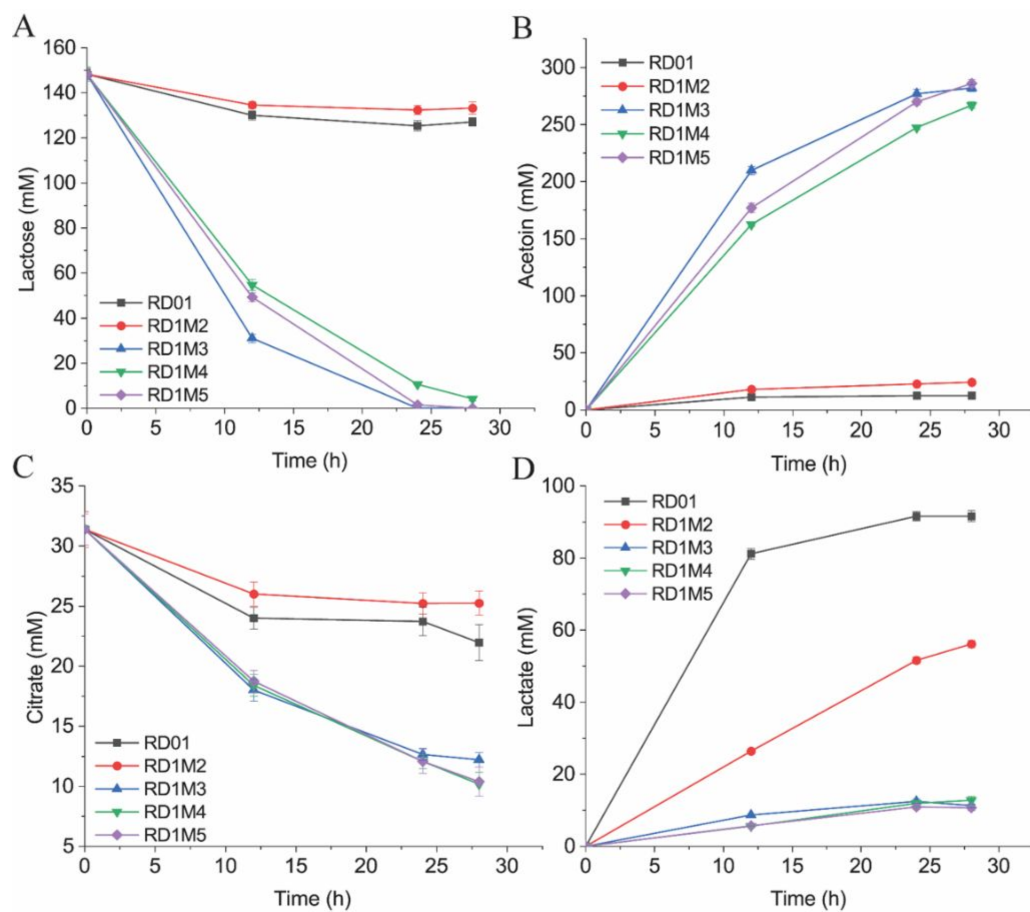


Figure 2.



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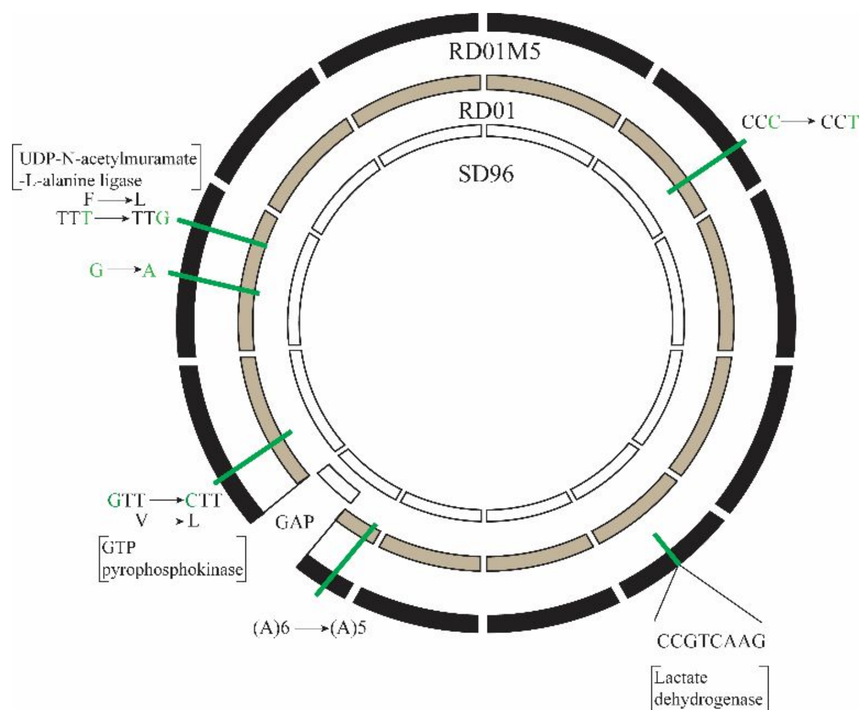


Figure 4.

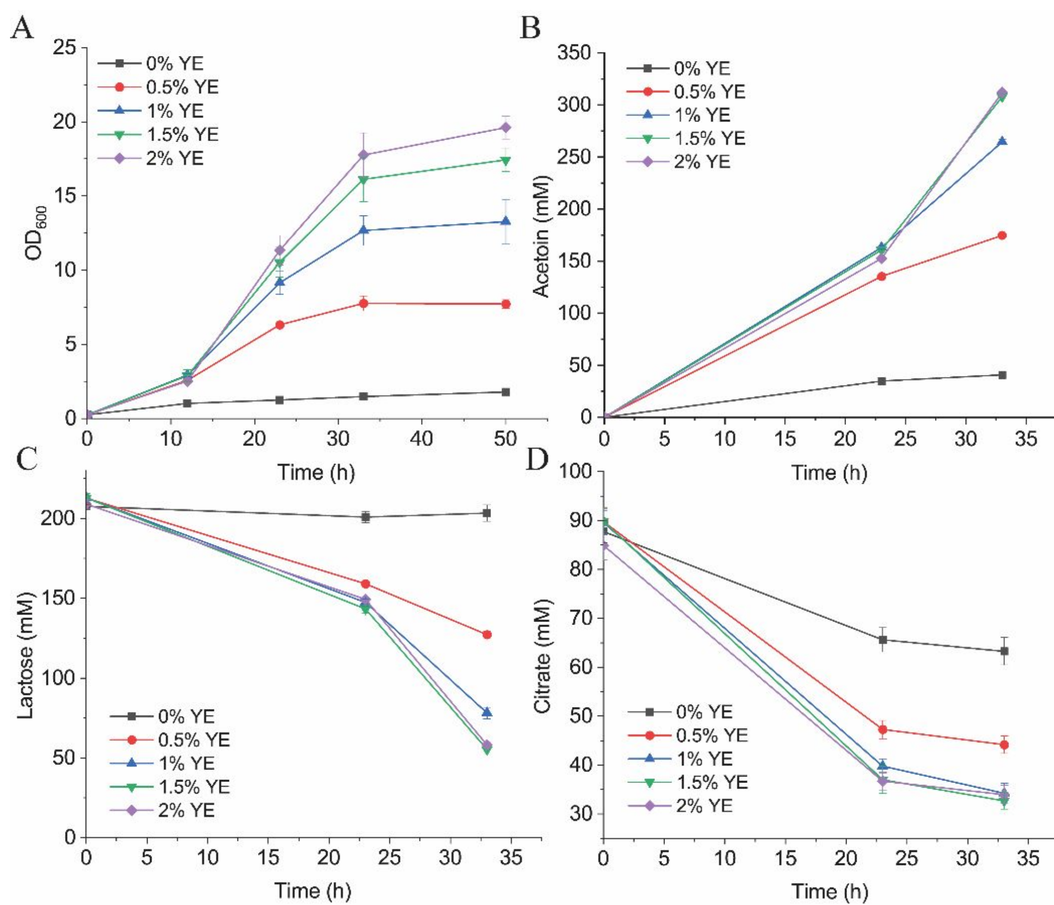
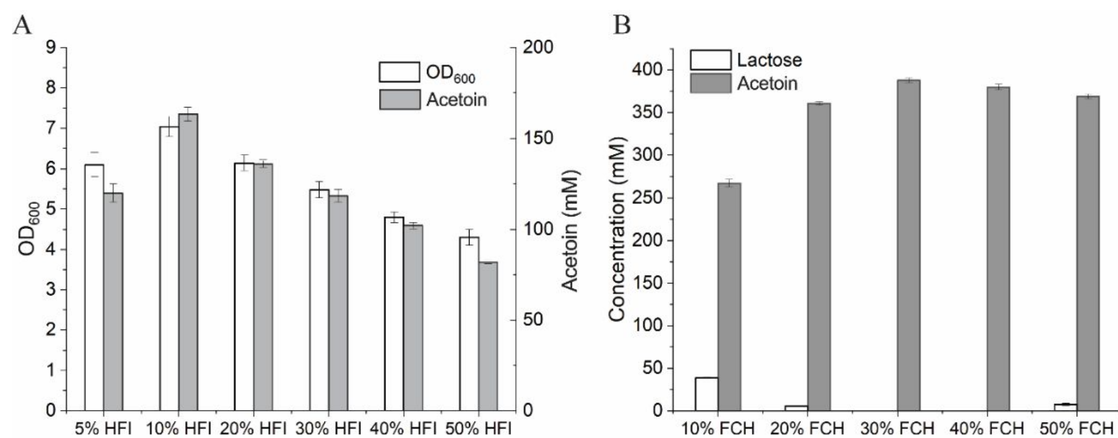


Figure 5.



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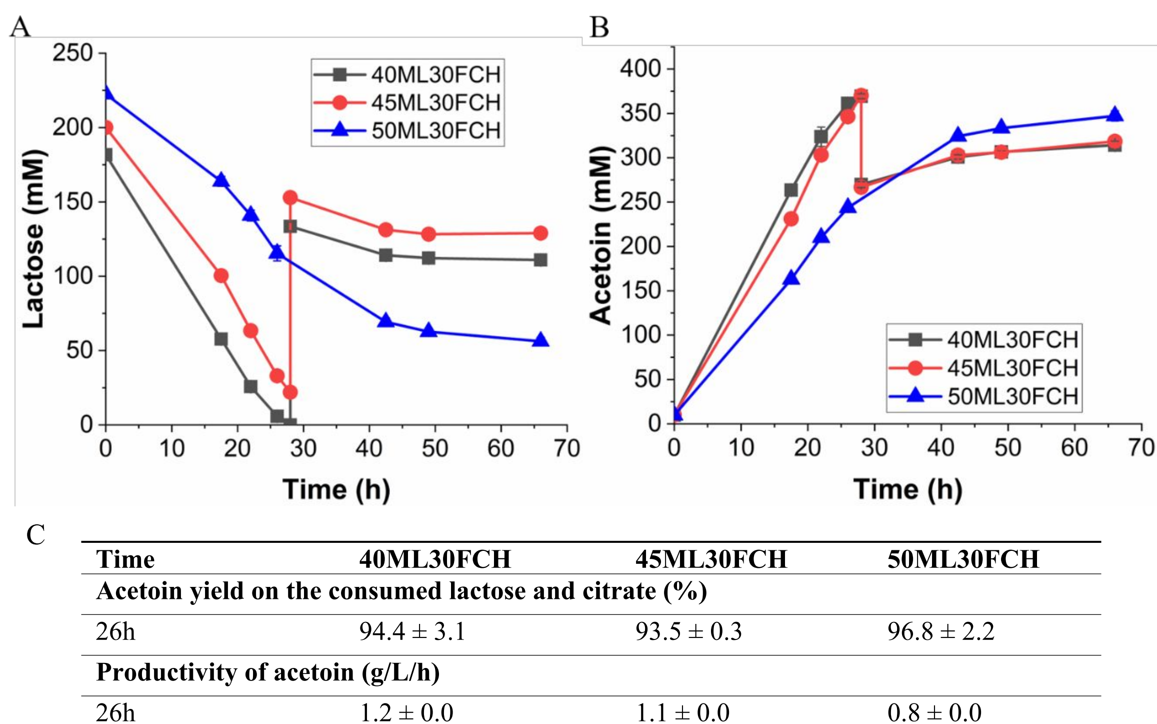
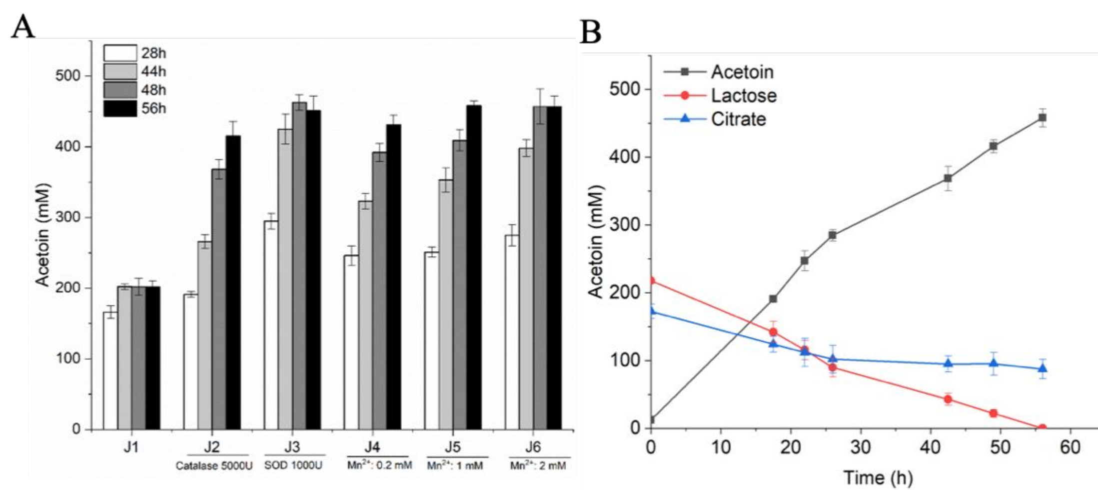


C

Time	40ML30FCH	45ML30FCH	50ML30FCH
Acetoin yield on the consumed lactose and citrate (%)			
26h	94.4 ± 3.1	93.5 ± 0.3	96.8 ± 2.2
Productivity of acetoin (g/L/h)			
26h	1.2 ± 0.0	1.1 ± 0.0	0.8 ± 0.0

Figure 7.



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