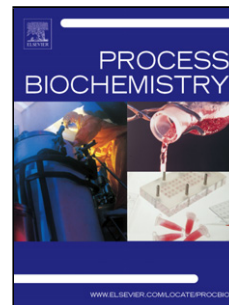# Accepted Manuscript

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Efficient production of acetoin by fermentation using the newly isolated mutant strain *Lactococcus lactis* subsp. *lactis* CML B4

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Graphical Abstract
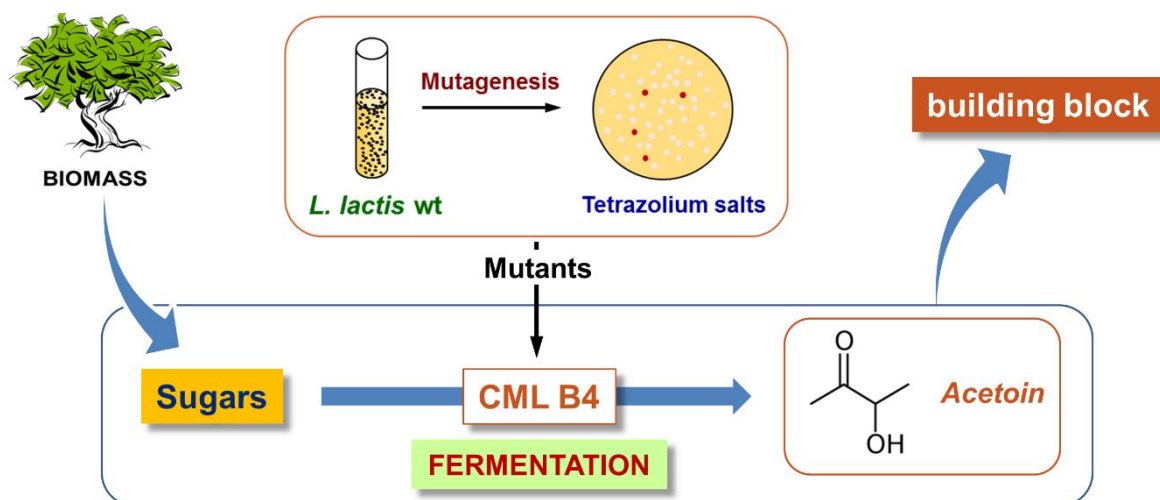
Highlights

- A new strain, *L. lactis* CML B4, overproducing acetoin was obtained by mutagenesis.
- This *ldh*-mutated strain showed reduced LDH and increased NOX activities.
- Fermentations yielded >40 and 59 gL^{-1} acetoin in batch and fed-batch, respectively.
- Maximum yields were close to 88% and productivities exceeded 2 gL^{-1}h^{-1}.
- Potential use of the strain for industrial production of acetoin from bioresources.

Abstract

With the aim of applying biotechnology to produce acetoin, a chemical that can be used as an aroma and as a building block for other compounds, several putative mutants with reduced lactic acid synthesis were obtained from a wild-type homolactic strain of *L. lactis* subjected to chemical mutagenesis. Among these mutants, a strain was isolated, CML B4, that showed reduced lactate dehydrogenase (LDH) and increased NADH oxidase (NOX) activities. Shaken flask cultures of this mutant strain mainly produced acetoin, increasing the levels produced compared to the wild-type strain by 15-fold. A point mutation detected in the *ldh* gene encoding LDH was probably the genetic defect responsible for this phenotype. In pH-controlled aerobic batch fermentation, the CML

B4 strain produced more than 40 gL^{-1} acetoin, which was increased by up to 59 gL^{-1} in fed-batch fermentations, with yields close to 88 and 74%, respectively, and productivities exceeding 2 gL^{-1}h^{-1}. These results indicate that this strain could be used industrially as a cell factory for the production of acetoin from bioresources.

Keywords: *Lactococcus lactis*; acetoin; fermentation; mutant; 1,3-butadiene

1. Introduction

Acetoin is currently a fine chemical that has attracted great interest for use as an aroma, with potential applications as an additive in the food, pharmaceutical and cosmetics industries. In addition, owing to the chemical structure of acetoin, which is small in size and has two different potentially reactive functional groups, it can also be used in the chemical industry as a building block for other compounds [1]. However, such uses require low cost, as it must be converted into a commodity.

Although acetoin can be produced by means of several chemical methods, their low selectivity (racemic mixtures containing both optical isomers are produced), harsh reaction conditions needed, and especially the need to use fossil feedstocks have led to a search for new alternative production methods that are more sustainable and use renewable feedstocks. An attractive alternative would be the use of biotechnology, as acetoin is produced as a result of the metabolic activity of a number of microorganisms, including some strains of lactic acid bacteria [2].

One of these chemicals that can use acetoin as a building block is 1,3-butadiene (BD), which has a worldwide market of 11 Mt/year, with its main application in the manufacturing of synthetic rubber. A large percentage of BD has been and is still

produced as a by-product of the manufacture of ethylene from naphtha. Approximately 16 kg of BD are produced per 100 kg of ethylene. In the past few years, ethylene producers have been shifting raw material from naphtha to C1 and C2 chemicals, such as ethane, leading to the production of 2 kg of BD per 100 kg of ethylene. The obvious consequence is that a BD shortage is expected. Consequently, new on-purpose processes for synthesizing BD are currently being developed [3], such as the Biosyncaucho[TM] process owned by Biosyncaucho S.L. and developed by TECNALIA (Fig.1), in which BD is obtained in a three-step process involving acetoin production by carbohydrate fermentation [4,5], hydrogenation [6], or electroreduction [7] to 2,3-butanediol and dehydration of the latter to BD. To make this process sustainable, the Biosyncaucho[TM] process preferably uses carbohydrates derived from lignocellulosic materials or industrial biowastes and byproducts.

Among lactic acid bacteria, the bacterium *Lactococcus lactis* is of special interest because it shows several advantageous features that make it a highly suitable host for the development of cell factories intended for efficient production of valuable industrial chemicals, such as acetoin, by fermentation. The metabolic pathway leading to acetoin synthesis has already been extensively studied in *L. lactis* [8,9]. It starts with pyruvate resulting from sugar metabolism and comprises two steps. In the first step, thiamine-dependent α-acetolactate synthase catalyzes the condensation of two molecules of pyruvate, yielding a molecule of α-acetolactate and releasing a molecule of CO_2. α-Acetolactate is then converted into acetoin by a decarboxylation reaction catalyzed by α-acetolactate decarboxylase. In addition, some strains of *L. lactis* subsp. *lactis*, all of them belonging to the biovar *diacetylactis*, can also synthesize acetoin through an alternative pathway from citrate [10].

Unfortunately, although *L. lactis* contains a complete metabolic pathway for acetoin biosynthesis, the natural production of this metabolite is generally very low. This is because the main pathway of carbohydrate metabolism in lactic acid bacteria leads to lactic acid synthesis through a reaction catalyzed by lactate dehydrogenase (LDH) [11]. However, there have been some attempts to increase the production of acetoin in *L. lactis* mainly using three different strategies. The first strategy involves the inactivation of LDH to block the main pathway of pyruvate metabolism and reroute this intermediate towards alternative metabolic pathways [8,12,13]. The second strategy focuses on overexpression of α-acetolactate synthase [12], the first enzyme of the acetoin biosynthesis pathway. The third strategy involves overexpression of NADH oxidase (NOX) as a method of modulating the cellular NADH/NAD$^+$ ratio, which was supposed to be the main factor regulating metabolic fluxes in *L. lactis* [9,14,15]. All of these efforts result in the production of modest levels of acetoin, with yields no higher than 30-35 mM. Although it is true that these procedures generally result in important relative increases in acetoin production, their absolute values are still insufficient for industrial use.

In this paper, we present a new method to convert *L. lactis* into an efficient acetoin producer with the potential for application in large scale production of this metabolite. This paper addresses the isolation of a new mutant strain of *L. lactis* that has an enhanced ability to produce acetoin under aerobic conditions and highlights its use in fermentation processes, leading to acetoin levels never attained in this bacterium.

2. Materials and methods

2.1. Microorganisms and culture media

All bacterial strains used in this work are derivatives of the *L. lactis* subsp. *lactis* NCIMB 702118 (previously known as NCDO 2118) wild-type strain. Shaken flask cultures were carried out in M17 medium [16], or YEC medium (5 gL^{-1} yeast extract, 20 mM sodium citrate buffer, pH 6.5), both containing 10 gL^{-1} glucose as a carbon source, at 30 °C and 250 rpm orbital shaking.

2.2. Random mutagenesis

A 100-mL Erlenmeyer flask containing 10 mL of M17 medium was inoculated with a single colony of *L. lactis* subsp. *lactis* NCIMB 702118 grown for three days on a plate of YEC agar medium and was cultured for 24 hours. Bacterial cells were washed twice by centrifugation/resuspension in 100 mM potassium phosphate buffer (pH 7.5) and finally resuspended in 1 mL of the same buffer. To this concentrated cell suspension, 120 μL of the chemical mutagen ethyl methanesulfonate (EMS) were added, and the resulting suspension was incubated at room temperature for 15 minutes with gentle shaking. Then, the cells were washed twice with 10 mL of the same potassium phosphate buffer, resuspended in 10 mL of M17 medium and incubated with shaking for one hour. This mutagenized bacterial suspension was either used directly or stored at −80 °C with 10% glycerol until further use.

2.3. Selection of mutant strains deficient in the biosynthesis of lactic acid

Low lactate-producing mutants were selected according to the procedure described by El Attar et al. [17], with some modifications. Selection was performed on agar plates containing modified M17 medium with only a 5% of its buffering capacity that was supplemented with 10 gL^{-1} 2,3,5-triphenyl tetrazolium. High lactate-producing bacteria

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growing in this selection medium formed pink colonies and it was expected that low

lactate producers would appear as red/brown colonies.

2.4. Determination of the metabolite concentrations

The acetoin, 2,3-butanediol, lactate, acetate, ethanol and glucose/sucrose concentrations

were measured by HPLC using an Aminex HPX-87H 300 x 7.8 mm (Bio Rad) column

and a Microguard Cation H Refill Cartridge precolumn, with the following conditions:

mobile phase, 0.01 N H_2SO_4; flow rate, 0.7 mL/min; column temperature, 55 °C. Peak

quantification was performed with a refractive index detector.

2.5. Cell extract preparation

L. lactis overnight cultures were harvested by centrifugation at 13,000 rpm for 15 min at

4 °C, washed twice in 50 mM sodium phosphate buffer (pH 7.0), and finally

resuspended in a 1/20 volume of the same buffer. An equal volume of acid-washed

glass beads was added to this bacterial suspension, and the mixture was vortexed in

three cycles of 30 sec at maximum speed interspersed with 1 min cycles of cooling on

ice. The resulting lysed cell suspension was centrifuged at 13,000 rpm for 15 min at 4

°C to remove cell debris and glass beads, and the cleared supernatant was recovered as

the cell extract.

2.6. Enzyme assays

The LDH activity was measured spectrophotometrically as described by Boumerdassi et

al. [8]. Assays were carried out at 30 °C in a reaction mixture containing 50 mM Tris-

maleate buffer (pH 7.0), 10 mM sodium pyruvate, 1 mM fructose-1,6-bisphosphate,

0.15 mM NADH, and 100 μL of cell extract. Appropriate blanks were also prepared

under the same conditions, but lacked the sodium pyruvate substrate to correct for

competing activities. One LDH unit was defined as the amount of enzyme that, under

the above assay conditions, catalyzed the conversion of 1 μmol of pyruvate to lactate in 1 min.

NOX activity assays were carried out at 30 °C in a reaction mixture containing 50 mM Tris-maleate buffer (pH 7.0), 0.15 mM NADH, and 100 μL of cell extract. One NOX unit was defined as the amount of enzyme that, under the above assay conditions, catalyzed the conversion of 1 μmol of NADH to NAD^+ in 1 min.

2.7. Measurement of the biomass concentration

Cell growth was measured spectrophotometrically based on the optical density at 600 nm (OD_{600}) and was correlated to the cell dry weight (in $mgmL^{-1}$) by means of a standard curve prepared from washed cell suspensions heated at 80 °C until constant weight.

2.8. Determination of the protein concentration

The protein concentrations in cell extracts were quantified by the Bradford assay [18] using lysozyme as the standard.

2.9. Gene sequencing

Genomic DNA of the CML B4 strain was isolated using the GenElute™ Bacterial Genomic DNA Kit (Sigma) according to manufacturer instructions. Sequencing of coding and promoter regions of the genes *ldh*, *noxE*, *ccpA* and operon *aldB-aldR* was carried out on this genomic DNA on both strands by automated capillary sequencing (3130xl Genetic analyzer ABI, Foster City, CA, USA), following standard procedures. The genome sequence of the wild-type strain *L. lactis* NCDO 2118 (=NCIMB 702118) (GenBank accession number CP009054) was used as a template for designing the sequencing primers and for the sequence analysis.

2.10. Fermentation procedure

Fermentations were carried out in double-concentrated M17 (2×M17) medium containing 100 gL^{-1} glucose as the carbon source. In some cases, this medium was substituted by other media composed of only yeast extract or yeast extract plus corn steep liquor (CSL), where 100 gL^{-1} sucrose was included instead of glucose. Batch fermentations were carried out in a Biostat B-plus fermenter (Sartorius) equipped with a 2-L reaction vessel and containing 1 L of medium. The fermentation temperature was set at 30 °C and mixing was achieved using a Rushton impeller operating at 500-750 rpm. The dissolved oxygen concentration was fixed at 30% with respect to the saturation concentration by supplying an air flow of 2 LL^{-1}min^{-1}. When, as a result of the increasing cell biomass, this air flow was not enough to maintain that dissolved oxygen concentration, pure oxygen was automatically injected into the culture to ensure that its value was never under 30%. The pH of the medium was kept constant at different values from experiment to experiment by the automatic addition of 5 M NaOH. Foam formation was controlled with the addition of 0.5 mLL^{-1} antifoam 204 (Sigma). In fed-batch fermentations (only with 2×M17 medium), after the first batch stage described above, an additional supply of 100 g of glucose was fed to the culture when the starting glucose was exhausted. Fermentations were started with a 2% (v/v) inoculum grown in either M17 medium or YEC medium.

3. Results and discussion

3.1. Isolation of mutant strains overproducing acetoin

The strategy to obtain new *L. lactis* strains overproducing acetoin was primarily based on the isolation of mutants deficient in the biosynthesis of lactic acid. Following mutagenesis of the wild-type strain *L. lactis* subsp. *lactis* NCIMB 702118, a total of 26,000 colonies were screened on selection medium to detect putative mutants. Four colonies showing an intense red/brown color, over the background of light pink colonies, were isolated and numbered from CML B2 to CML B5 and were preserved frozen at −80 °C in 10% (v/v) glycerol for further analysis.

First, the metabolite production by wild-type and mutant strains grown aerobically (shaken flask cultures) in M17 medium was assessed (Fig. 2A). All mutant strains except CML B2 displayed an increased production of acetoin and a reduced synthesis of lactic acid, well above and below, respectively, the levels of the wild-type strain. Regarding these features, the CML B4 mutant produced the most acetoin and least lactic acid, with values 15 times higher and 12.5 times lower, respectively, than those produced by the wild-type strain. It is worth noting that, contrary to the wild-type strain, all of the mutants were able to produce detectable levels of 2,3-butanediol, especially the CML B3 mutant (up to 14 mM). Other metabolites, such as acetic acid and ethanol, were produced in minimal quantities.

Mutant strains showed higher cell growth (biomass formation) than the wild-type strain, again with the exception of mutant CML B2 (Fig. 2B). For the first 5 h of culture growth kinetics were identical for all the strains but CML B3, which showed a reduced growth rate. From 5 h wild-type and CML B2 mutant stopped growing, while biomass of strains CML B4 and B5 increased still further. Cell growth correlated with lactic acid

production and the resulting pH decrease in the culture medium; as lactic acid production decreased, there was less resulting acidification and higher cell growth was obtained (compare Figs. 2A and 2B).

Next, the LDH and NOX activities of the cell extracts from the isolated mutants were measured and compared with the values displayed by the wild-type strain. As expected, all four mutants showed, to a greater or lesser extent, a LDH activity lower than that of the wild-type strain (Fig. 3). Among them, the CML B4 strain was especially interesting because its LDH activity was only a 7% of that present in the wild-type strain. On the other hand, all of the mutants showed a strong increase in their NOX activities, ranging from a four- to 16-fold increase over that of the wild-type strain (Fig. 3). Decreased LDH activities were associated with increased NOX activities, generally resulting in a shift in the metabolite profile from lactic acid to acetoin.

All of these mutants were found to be true *L. lactis* bacteria derived from the wild-type strain used, as confirmed by phenotypic profiling and bacterial restriction endonuclease analysis (BRENDA) (results not shown). As strain CML B4 was the best acetoin producer, phenotypic stability tests were conducted to show its suitability as an acetoin production host. There were no significant differences (results not shown) in acetoin production and cell growth throughout twelve consecutive subcultures. Based on all of the above results, strain CML B4 was selected for further studies.

Following sequencing of several candidate target genes directly involved in the synthesis of acetoin, including *ldh*, *noxE*, *aldBR* and *ccpA*, encoding the L-LDH, NOX, the α-acetolactate decarboxylase operon and the carbon catabolite control protein CcpA, respectively, a point mutation was detected located in the *ldh* gene: an A to C change at position 182 of the nucleotide sequence (A182C), which resulted in the substitution of a

lysine by a threonine at position 61 of the amino acid sequence (K61T). No additional mutations were detected, so suggesting that this was the genetic defect that resulted in acetoin overproduction and lactic acid underproduction in the CML B4 strain.

The mechanism by which this point mutation would negatively affect LDH activity remains unknown, because the lack of identified functional motifs in that area of the LDH protein sequence, as far as we know. Determining whether the change of a positively charged lysine residue by an uncharged threonine residue could somewhat affect the binding of some substrate, co-factor or effector or, alternatively, destabilize the enzyme structure, leading to a reduced catalytic activity, would deserve further efforts.

It has not been surprising to find that the genetic defect carried by the CML B4 strain is located in the *ldh* gene, as previous studies already reported that inactivation of LDH to block the main pathway of pyruvate metabolism resulted in an enhanced production of acetoin [8,12,13]. It is well known that the key factor regulating pyruvate partitioning between different metabolic pathways in *L. lactis* is the cell redox potential [11,14,19], expressed as the $NADH/NAD^+$ ratio. When a metabolizable carbohydrate is present with a high glycolytic flux and under anaerobiosis, the $NADH/NAD^+$ ratio is high and pyruvate is solely converted into lactic acid by the *ldh*-encoded LDH. Therefore, a partial LDH inactivation would be expected to hardly affect metabolism under anaerobiosis, resulting in a homolactic fermentation pattern, as seen with the CML B4 strain. Under aerobiosis, however, the situation is somewhat different because an additional enzyme, NOX, is also involved. Water-forming NOX, encoded by the *noxE* gene, catalyzes the oxidation of NADH to NAD^+ at the expense of oxygen, which is reduced to H_2O [14,20]. Therefore, in the presence of oxygen, NOX competes with

LDH for NADH, resulting in a decrease in the NADH/NAD$^+$ ratio and, consequently, in a lower LDH activity. In this situation, alternative metabolic pathways are activated, including acetoin formation, resulting in a shift towards mixed-acid fermentation. A defect in the *ldh* gene, as seen in the CML B4 strain, would shift NADH partitioning even more towards the NOX enzyme, likely enhancing acetoin synthesis.

The observed increase in NOX activity would also result from the decreased NADH/NAD$^+$ ratio, through an enhanced expression of the *noxE* gene mediated by the transcriptional regulators Rex and Spx in response to redox conditions, as occurs in *Streptococcus mutans* [21]. The genes coding for these redox-sensing regulators, *rex* and *trmA*, are also present in *L. lactis* [22], opening the possibility of such a kind of control.

3.2. Acetoin production by fermentation of the CML B4 strain in 2×M17 medium

It has long been known that *L. lactis* can produce acetoin. However, there have been no reports describing the high-scale production of acetoin using this bacterium in spite of its great potential as a cell factory.

The use of strain CML B4 in an industrial process would require performing high-scale cultures using carbon sources at high concentrations to maximize acetoin production and render a cost-efficient process. Such high-carbon media (e.g., 100 gL^{-1} glucose) are also associated with a need to increase the nitrogen source concentration (double-concentrated M17 medium) to maintain balanced growth. It is worth noting that acetoin synthesis was found to be growth-linked (see Fig. 4) and a precocious growth arrest occurred under nitrogen limitation, which resulted in cessation of acetoin production and conversion of the remaining glucose into lactic acid (results not shown). This

relationship between growth and acetoin production was also previously reported for *Listeria monocytogenes* [23].

Preliminary cultures were carried out in shaken flasks with concentrated media, but they were unsuccessful owing to insufficient pH control and oxygen supply in that type of culture. Consequently, further fermentations were performed using fermenters with the aim of avoiding the above problems and allowing cultures to be carried out under appropriate conditions.

Acetoin synthesis by the mutant strain CML B4 is an aerobic process, as was confirmed in the early fermentation experiments using three different dissolved oxygen concentration values: 15, 30 and 50%. It was necessary to maintain a minimum dissolved oxygen concentration of at least 30% to obtain the highest carbohydrate conversion into acetoin. At 15%, acetoin production decreased by 20% and that of lactic acid increased accordingly. Lower dissolved oxygen concentrations led to increasingly greater lactic acid synthesis and less acetoin production, resulting finally in a homolactic fermentation under anaerobiosis. Therefore, the subsequent fermentations were performed while maintaining the dissolved oxygen concentration at 30%, a value that ensured fully aerobic growth.

Next, a series of fermentations were carried out at different pH values in double-concentrated M17 medium with an initial high glucose content (100 gL^{-1}) to test the suitability of the mutant strain under industrial-like culture conditions. These fermentations were performed at 30 °C, the optimal growth and acetoin production temperature for the strain, with a stirring rate of 500 rpm. As seen in Table 1, acetoin production greatly depended on the culture pH, showing maximal production in the range of 6.5-7.0 (37.4 gL^{-1} at pH 6.5) and further decreasing towards more acidic

values. The acetoin yield reached a maximum value of 0.39 g/g of glucose,
approximately 80% of the maximum theoretical yield, and the productivity was slightly
above 1.5 $gL^{-1}h^{-1}$. Lactic acid was the main alternative metabolite, and its production
was in opposition to that of acetoin: the maximum acetoin concentration correlated with
the minimum lactic acid levels, and vice versa. There was limited 2,3-butanediol
synthesis at all pH values, although it tended to be slightly higher under acidic
conditions.

When the stirring rate was increased from 500 to 750 rpm, the acetoin concentration
increased by nearly 10% (up to 40.6 gL^{-1}) and the lactic acid concentration decreased
by 30% (Table 1). The acetoin yield and productivity also improved accordingly, with
the former reaching a value close to 90% of the maximum theoretical yield and the
latter surpassing 2 $gL^{-1}h^{-1}$. This positive effect is explained by the rise in the rate of
oxygen transfer from the gas phase to the liquid caused by the increased stirring rate
[24], allowing for a higher oxygen availability and, therefore, consumption rate by
bacteria. The time-course of a batch fermentation under the above optimal culture
conditions is shown in Fig. 4.

Finally, several attempts were made to perform fed-batch fermentations under
conditions providing the best results for the batch in terms of acetoin production (pH 6.5
and 750 rpm). The acetoin concentration reached a maximum value of 671 mM (59
gL^{-1}).

Several groups have previously performed studies of different ways to increase acetoin
production in *L. lactis* through metabolic engineering [8,9,12,13,14,15], preferably
involving LDH inactivation, or overexpression of α-acetolactate synthase or NOX.
Some of these efforts were reported to result in a comparative increase in acetoin

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production. However, the levels produced by these strains were still very modest, not higher than 30-35 mM, with yields reaching an average value close to 50-60%. To date, the two best attempts reported to produce higher concentrations of acetoin using *L. lactis* were based on strategies different to the above mentioned ones. In one of them, the supplementation of the culture medium with hemin or Cu^{2+}, resulted in the production of up to 105 mM acetoin [25]. Heme allows *L. lactis* to grow via respiration in the presence of oxygen [26], displacing metabolism from lactic acid synthesis to acetoin production [27]. In the other one, up to 157 mM acetoin were produced by introducing the ATP hydrolyzing F_1-ATPase and modulating its activity, as a result of the decreased ATP availability in the engineered strain [28]. Nevertheless, these acetoin productions still fall well below the levels reached in this work using the CML B4 strain.

3.3. Acetoin production in batch fermentation by the CML B4 strain in simplified media

M17 is a very complex and expensive medium, and therefore, it is unsuitable for cost-effective industrial production of acetoin. A simpler and cheaper medium was clearly needed. Initially, the possibility of reducing the number of ingredients in the medium was evaluated, and apart from the carbon source, a medium composed of only yeast extract was found to be sufficient to sustain growth and acetoin production similar to those achieved with M17 medium. Yeast extract, at concentrations higher than 30 gL^{-1}, was able to supply all of the nitrogen, vitamins and minerals required by the bacterium, greatly simplifying the composition of the medium. In addition, carbon sources other than glucose were also found to be suitable for acetoin fermentation. For example,

sucrose was as effective a carbon source as glucose, which opened the door to the use of cheap feedstocks, such as molasses.

Batch fermentations were then carried out using a simple culture medium composed of only yeast extract and 100 gL^{-1} sucrose, resulting in the production of 426 and 438 mM acetoin at yeast extract concentrations of 30 and 40 gL^{-1}, respectively, which are close to the values previously obtained with glucose containing 2×M17 medium.

Unfortunately, yeast extract is still a high-priced culture medium ingredient and must be used carefully in industrial fermentations because the production costs would be comparatively too high. Therefore, it was necessary to seek alternative substrates that would allow a performance similar to that achieved with yeast extract, but at a lower cost.

In line with the above statement, it could be seen that some of the yeast extract in the medium could be successfully replaced with CSL, without reducing acetoin production and even slightly increasing it. A batch fermentation in sucrose medium containing 30 gL^{-1} yeast extract and 20 gL^{-1} CSL yielded 473 mM (41.7 gL^{-1}) acetoin.

Further research in developing a low cost fermentation medium is currently ongoing, as it is considered to be a priority to attain a more cost-effective process for the biotechnological production of acetoin.

3.4. Comparison of *L. lactis* CML B4 with other acetoin producing bacteria

To the best of our knowledge, the *L. lactis* CML B4 strain described in this work is, by far, the *L. lactis* strain showing the highest acetoin production ever reported. In addition, it is also comparable to the best producers known among other bacteria. The previous findings obtained for the main acetoin-producing bacteria reported in the literature are summarized in Table 2, where they are compared to the results obtained in this work

with the *L. lactis* CML B4 strain.

As seen, the *L. lactis* CML B4 strain shows one of the highest acetoin productions and, interestingly, the highest acetoin yield reported to date: 88% in glucose batch fermentations. In addition, the acetoin productivities achieved with this strain are among the highest reported, only surpassed by one bacterium, *Enterobacter cloacae*. However, it is important to note that this bacterium is classified as a Risk Group 2 (RG2) pathogenic bacterium (as is *Serratia marcescens*, another of the best acetoin producers), whereas *L. lactis* is a safe RG1, GRAS bacterium. Therefore, the acetoin productivities shown by the *L. lactis* CML B4 strain can be considered to be the highest ever reported among non-pathogenic bacteria. This point has important implications because the use of pathogenic bacteria in industrial-scale fermentations is a cause of strong concern since appropriate and strict biosafety and containment measures must be adopted, significantly increasing production costs. This problem does not exist with a bacterium such as *L. lactis*, which can even be used in food applications, supporting its safe nature.

4. Conclusions

A new mutant strain of *L. lactis* overproducing acetoin was obtained following a strategy of classical mutagenesis and selection in a single step. This mutant strain efficiently converts carbohydrates into acetoin, with titers, yields and productivities comparable and even higher than those achieved by the best producers reported to date and with the additional advantage of being a safe, non-pathogenic bacterium. These characteristics suggest that this strain might be used industrially as a cell factory for the production of acetoin, a potential platform chemical derived from biomass.

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Figure captions

Fig. 1. The Biosyncaucho^{TM} process to produce 1,3-butadiene using acetoin as an intermediate.

Fig. 2. Metabolite production (A) and growth kinetics (B) of wild-type (wt) and putative mutant strains of *L. lactis*. Bacteria were grown aerobically (shaken flask cultures) in M17 medium containing 10 gL^{-1} glucose as a carbon source, at 30 °C and 250 rpm orbital shaking. Final pH values (pH$_f$) reached are indicated by dots in panel A.

Fig. 3. LDH and NOX activities in cell extracts from wild-type (wt) and putative mutant strains of *L. lactis*. Cell extracts, obtained from overnight cultures in M17 medium containing 10 gL^{-1} glucose at 30 °C and 250 rpm orbital shaking, and enzyme assays were carried out as explained in the Materials and Methods section.

Fig. 4. Time-course of a batch fermentation by *L. lactis* CML B4. Culture was carried out in 2×M17 medium containing 10% glucose under optimal conditions: pH 6.5, 30 °C, 30% dissolved oxygen concentration, 750 rpm stirring.
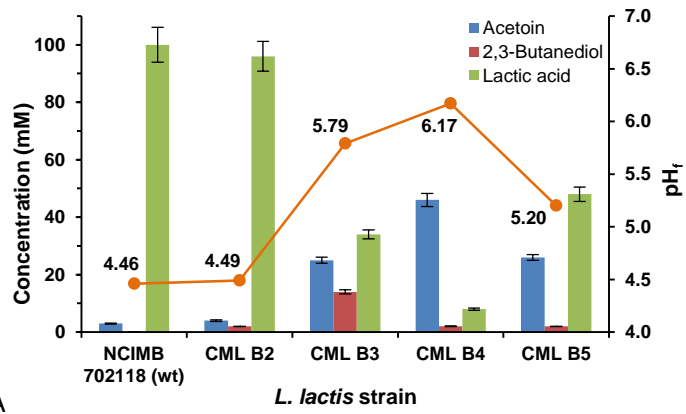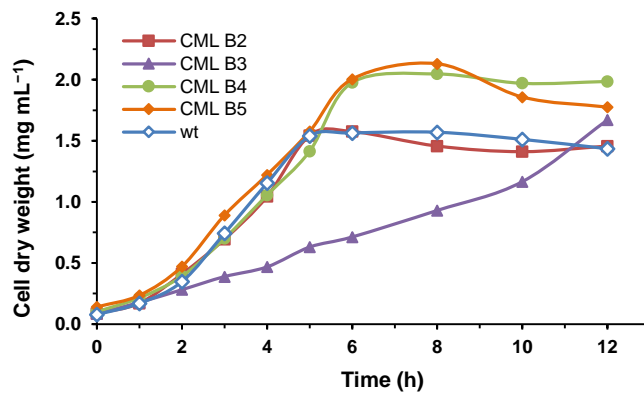
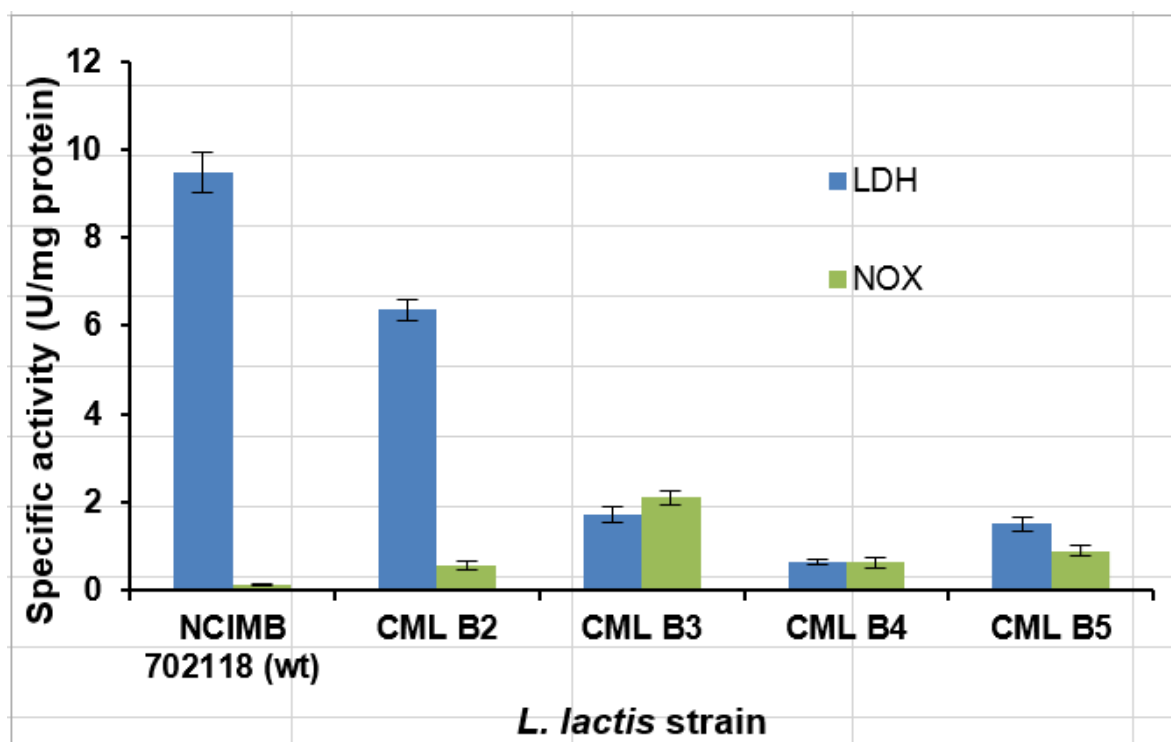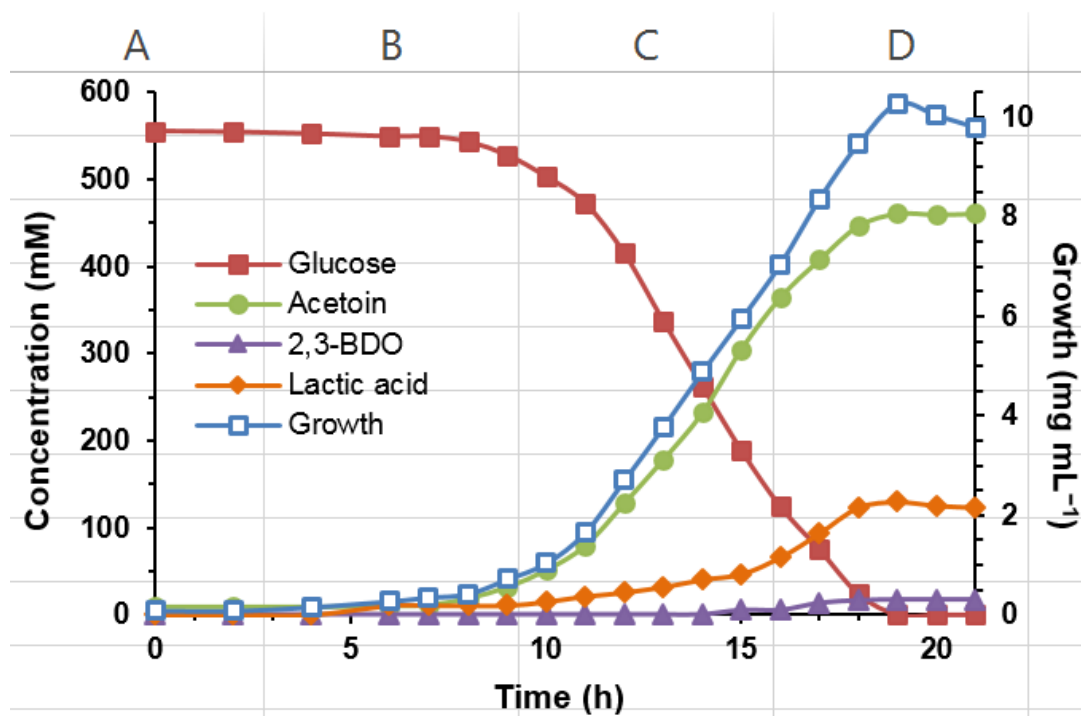Figr-1
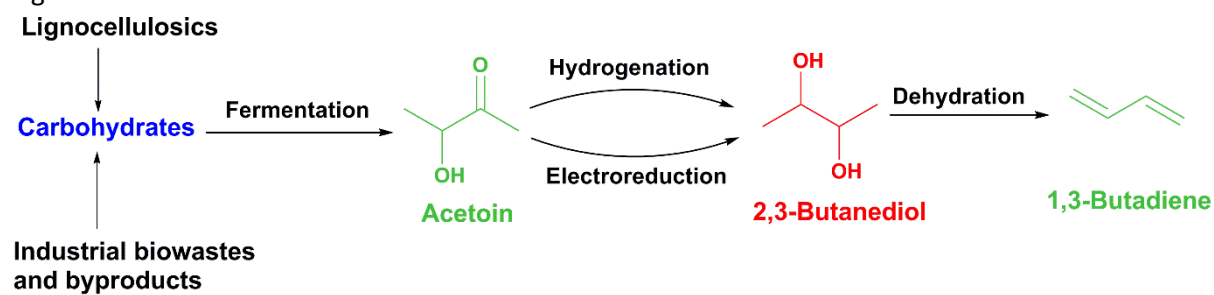
Fig 2A

Fig 2B

Fig 3

Fig 4

Table 1. Metabolite production by the *L. lactis* CML B4 mutant strain under aerobic fermentation conditions in 2×M17 medium. In all fermentations, the initial glucose concentration was 100 gL^{-1}. In fed-batch fermentations, after the first batch step, an additional supply of 100 g of glucose was fed to the culture when the starting glucose was exhausted. Fermentations were carried out as explained in the Materials and Methods section. Yields are expressed in g of acetoin/g of glucose. Carbon recovery is calculated based on detected metabolites.

pH	Stir rate (rpm)	Mode	Acetoin			2,3-BDO C (mM)	Lactic acid C (mM)	Carbon recovery (%)
			C (mM)	Y (gg^{-1})	P (gL^{-1}h^{-1})			
7.0	500	B	398 ± 26	0.37 ± 0.02	1.46 ± 0.09	23 ± 1.0	120 ± 6	86.7 ± 7
6.5	500	B	425 ± 27	0.39 ± 0.02	1.56 ± 0.10	29 ± 1.4	189 ± 12	98.8 ± 9
6.0	500	B	298 ± 15	0.30 ± 0.01	1.14 ± 0.06	38 ± 1.9	287 ± 17	86.4 ± 9
5.5	500	B	260 ± 13	0.25 ± 0.01	1.00 ± 0.05	51 ± 3.1	343 ± 16	86.9 ± 9
6.5	750	B	461 ± 31	0.43 ± 0.03	2.03 ± 0.14	17 ± 0.9	130 ± 9	97.8 ± 9
6.5	750	F-B	671 ± 41	0.36 ± 0.02	2.11 ± 0.13	76 ± 2.5	356 ± 21	83.3 ± 8

B, batch; **F-B**, fed-batch; **2,3-BDO**, 2,3-butanediol; **C**, concentration; **Y**, yield; **P**, productivity.

Table 2. Fermentative acetoin production by different bacteria.

Microorganism	Mode	Substrate	Acetoin concentration (gL^{-1})	Acetoin yield (%)*	Acetoin productivity $(gL^{-1}h^{-1})$	Reference
B. subtilis	Batch	Glucose	76.0	86.0	1.00	[29]
B. pumilus	Batch	Glucose	63.0	64.4	1.05	[30]
B. amyloliquefaciens	Batch	Glucose	71.5	84.4	1.63	[31]
B. licheniformis	Batch	Glucose	41.3	84.4	1.14	[32]
P. polymyxa	Fed-batch	Glucose	55.3	75.6	1.32	[33]
E. cloacae	Fed-batch	Glucose	55.2	76.3	2.69	[34]
S. marcescens	Fed-batch	Sucrose	75.2	69.9	1.88	[35]
L. lactis CML B4	Batch	Glucose	40.6	88.0	2.09	This work
L. lactis CML B4	Batch	Sucrose	41.7	85.7	2.20	This work
L. lactis CML B4	Fed-batch	Glucose	59.1	74.0	2.11	This work

* Yield is expressed as % of the maximum theoretical one, which is 0.49 g g^{-1} for glucose and 0.51 g g^{-1} for sucrose.