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Superior anodic electro-fermentation by enhancing capacity for extracellular electron transfer

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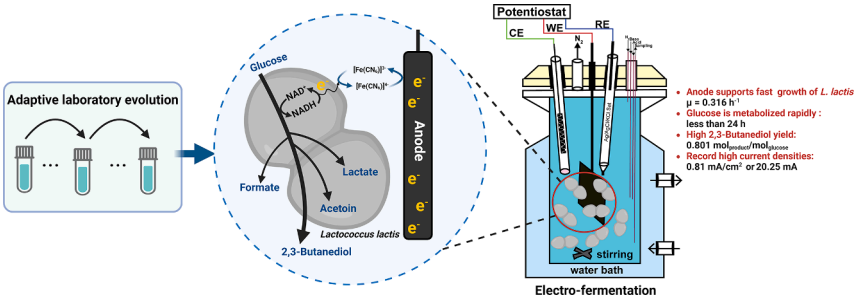
HIGHLIGHTS

- First demonstration of AEF supported growth of *Lactococcus lactis*.
- ALE is a promising approach for obtaining mutants with enhanced capacity for AEF.
- Record high current densities achieved (0.81 ± 0.05 mA/cm^2)
- 2,3-Butanediol produced with high yield and productivity.

GRAPHICAL ABSTRACT



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ABSTRACT

Anodic electro-fermentation (AEF), where an anode replaces the terminal electron acceptor, shows great promise. Recently a *Lactococcus lactis* strain blocked in NAD^+ regeneration was demonstrated to use ferricyanide as an alternative electron acceptor to support fast growth, but the need for high concentrations of this non-regenerated electron acceptor limits practical applications. To address this, growth of this *L. lactis* strain, and an adaptively evolved (ALE) mutant with enhanced ferricyanide respiration capacity were investigated using an anode as electron acceptor in a bioelectrochemical system (BES) setup. Both strains grew well, however, the ALE mutant significantly faster. The ALE mutant almost exclusively generated 2,3-butanediol, whereas its parent strain mainly produced acetoin. The ALE mutant interacted efficiently with the anode, achieving a record high current density of 0.81 ± 0.05 mA/cm^2. It is surprising that a Lactic Acid Bacterium, with fermentative metabolism, interacts so well with an anode, which demonstrates the potential of AEF.

1. Introduction

Aerated bioreactors are used to culture a wide range of important industrial microorganisms, including those used in food fermentations (Kirsop, 1974; Koebmann et al., 2008; Pedersen et al., 2012; Suttikul et al., 2023). However, the aeration process requires a high energy input and thus the running costs are high, often limiting the extent of scaling up of the bioreactors. The low solubility of oxygen in aqueous solutions

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makes it challenging to achieve a high volumetric gas–liquid coefficient (i.e. K_La) and dissolved oxygen (DO) level (Doran, 1995), which are important for cell growth and a high yield of the desired fermentation products (Lv et al., 2020). Common ways to improve DO level include costly measures e.g. high stirring speed, high gas flow rate (of even pure oxygen), high bioreactor pressure, etc. (Garcia-Ochoa and Gomez, 2009). Other challenges associated with aeration are foaming problems (Delvigne and Lecomte, 2010) and the strong oxidative stress imposed on the microorganisms (Cesselin et al., 2011; Gibson et al., 2008; Li et al., 2011). Nevertheless, oxygen still remains the most commonly used terminal electron acceptor, mainly due to lack of suitable alternatives and its relatively low cost.

The facultative anaerobic bacterium, *Lactococcus lactis*, is an important food microorganism and cell factory for producing food ingredients such as butter aroma, vitamins and nisin (Liu et al., 2020; Sybesma et al., 2004; Zhao et al., 2021). In the culture industry, aerobic conditions are widely used to suppress lactate production, as lactate lowers pH and inhibits cell growth (Sano et al., 2020). In the presence of oxygen, NADH oxidase can oxidize NADH and change *L. lactis* from a homolactic bacterium to an efficient acetoin-producing bacterium (Lopez de Felipe et al., 1998). Besides, some *L. lactis* strains are capable of respiring when either heme, hemin, or protoporphyrin IX is available, which has beneficial effects on growth and biomass yield, why culture manufacturers often harness respiration when culturing of *L. lactis* (Duwat et al., 2001; Lechardeur et al., 2011; Rezaïki et al., 2004). However, as mentioned above aerated culturing is associated with some challenges, and furthermore the use of animal blood, as a source of heme, in microbial food cultures can be unwanted as well. Under anaerobic conditions, although the oxygen is avoided, the type of product is also limited because the regeneration of NAD^+ is mainly achieved by lactate dehydrogenases, and typically 90% of the metabolized sugar ends up as lactate (Nordkvist et al., 2003). Therefore, by replacing oxygen with an alternative electron acceptor it may be possible to alter the fermentation product composition by affecting the redox balance, while at the same time avoiding some of the challenges associated with oxygen.

Recently, a mutant of *L. lactis* blocked in NAD^+ regeneration, CS4363, was demonstrated to grow in the absence of oxygen, when ferricyanide was used as electron acceptor, and extracellular electron transfer (EET) could be enhanced by adaptive laboratory evolution (ALE) (Gu et al., 2023). In that study, a high concentration of ferricyanide was used (50 mM), and ferricyanide was not regenerated. If the electron acceptor could be regenerated that it would be a great advantage, as this would allow lower concentrations to be used. Anodic electro-fermentation (AEF) could be the solution, where an anode accepts electrodes either directly from living cells or via mediators (Moscoviz et al., 2016; Vassilev et al., 2021; Virdis et al., 2022). The use of AEF to produce important chemicals has been systematically reviewed for other microorganisms previously (Gong et al., 2020), and has great potential. AEF enabled high-yield production of 2-keto-gluconate by the obligate aerobe *Pseudomonas putida* under anoxic conditions in a bioelectrochemical system (BES) setup (Lai et al., 2016). AEF was able to boost the cellular energy supply and promote growth and production of L-lysine by *Corynebacterium glutamicum* (Vassilev et al., 2018). For another important industrial bacterium, *Bacillus subtilis*, AEF was shown to alter cofactor levels and enhance acetoin production under limited aeration conditions (Sun et al., 2023). A redox imbalance was also overcome in the production of 3-hydroxypropionic acid by *Klebsiella pneumonia* by using a BES (Kim et al., 2017).

This study aimed to investigate the performance of CS4363 and its adapted version in a BES where an anode functions as the electron sink. Hence, for the two strains, growth, product formation and the efficiency of interaction between strains and anode were characterized and compared under the electrochemical cultivation conditions with endogenous or exogenous mediator.

2. Material and methods

2.1. Strains and cultivation conditions

In this study, the mutant *L. lactis* CS4363 (*L. lactis* MG1363 Δ^3*ldh Δpta ΔadhE*) (Solem et al., 2013) and *L. lactis* CS4363-F2 (*L. lactis* CS4363 adapted on ferricyanide about 600 generations) (Gu et al., 2023) were used. Cells were cultivated in a customized defined medium (SALN) (29), modified from the SA medium designed by Jensen et al (Jensen and Hammer, 1993). The changes included: i) replacing the MOPS buffer with disodium-β-glycerophosphate buffer, since MOPS buffer was found to interfere with the quantification of acetoin; ii) adding six nucleosides (20 mg/L adenosine, 20 mg/L guanosine, 20 mg/L cytidine, 20 mg/L thymidine, 20 mg/L inosine, 20 mg/L uridine) and iii) adding 2 mg/L lipoic acid (cofactor for pyruvate dehydrogenase complex) in the final recipe. Glucose was used as the sole carbon source in all experiments. SALN medium was filtered using rapid-flow^{TM} sterile disposable bottle top filter (0.2 μm pore size, Thermo Scientific, USA).

To prepare pre-cultures, a single colony from M17 agar plates (Thermo Fisher Scientific, USA) with 1% glucose was inoculated into 25 ml SALN medium with 1% glucose in 250 ml shake flasks and incubated at 30 °C, 150 rpm for overnight. Then 1 % overnight culture was transferred into fresh SALN medium with 1% glucose. The cells were harvested by centrifugation (7000 *g*, 5 min, 20 °C) when the cell density reached $OD_{600} = 0.2$ (log phase), and then re-suspended in fresh SALN medium with 0.5% glucose for further fermentation experiments in bioreactors.

2.2. Cyclic voltammetry of different media

Cyclic voltammetry (CV) tests were recorded by using a potentiostat (Autolab PGSTAT12, EcoChemie, Netherlands) in a three-electrode setup with a $Ag/AgCl/KCl_{sat}$ as the reference electrode, a platinum wire counter electrode, and a polished glassy carbon working electrodes (GCE, diameter: 0.4 cm) respectively. CVs of M17 and SALN medium with 1% glucose were recorded with a scan rate of 5 mV/s. Dissolved oxygen was removed by bubbling argon gas through the medium before and flushing the headspace with argon throughout the measurements. Detailed figure of this setup is provided (see Supplementary Materials).

2.3. Bioelectrochemical system setup

The construction and setup of the bioelectrochemical system (BES) were as previously described (Lai et al., 2019, 2016). Briefly, pre-treated carbon cloth (projected surface area of 25 cm^2) was applied as the working electrode, and stainless steel mesh was used as the counter electrode. The potential of the working electrode was poised at 0.5 V versus $Ag/AgCl/KCl_{sat}$ using a potentiastat (VMP3, Bio-Logic, USA). Potassium ferricyanide (Sigma-Aldrich, USA) with a final concentration of 5 mM was added to the working chamber as electron transfer mediator. Anaerobic conditions throughout the experiments were assured by bubbling the culture medium with nitrogen (20 ml/min). The volume of medium in the BES reactor was 320 ml. The BES reactors were kept at 30 °C using a circulator water batch, and the electrolytes in the working chamber were mixed at 400 rpm using magnetic stirring (25 × 25 × 9 mm cross-shape stir bar). All redox potentials in this manuscript were reported against the $Ag/AgCl/KCl_{sat}$ reference electrode, unless specified.

2.4. Analytics and sampling

The concentrations of glucose and other *exo*-metabolites (acetoin, 2,3-butanediol, formate, lactate, pyruvate) were determined by high-performance liquid chromatography (HPLC) using an Agilent Hiplex H column (300 × 7.7 mm, PL1170-6830, Santa Clara, CA, USA). 3 mM H_2SO_4 was used as the mobile phase at a flow rate of 0.4 ml/min. The

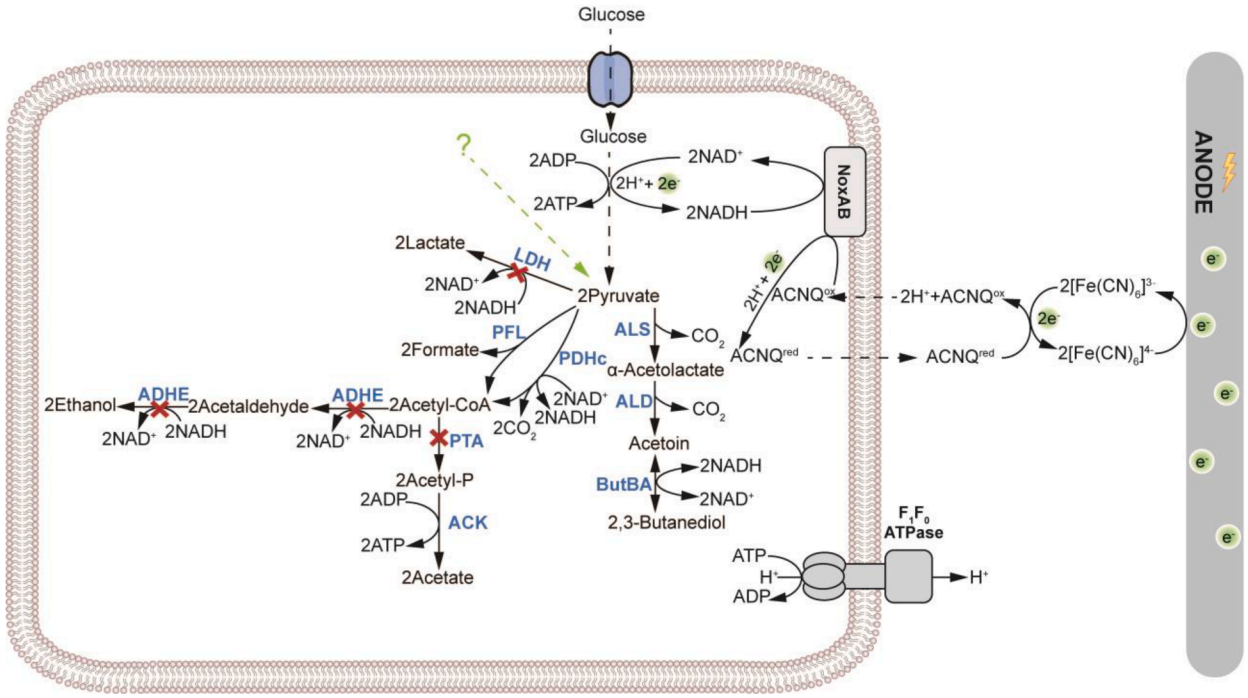

Fig. 1. Metabolism of *L. lactis* in anode compartment of the BES reactor. LDH: lactate dehydrogenases; ALS: α-acetolactate synthase; ALD: α-acetolactate decarboxylase; ButBA: 2, 3-butanediol dehydrogenase; PDHc: pyruvate dehydrogenase complex; PFL: pyruvate-formate lyase; ADHE: acetaldehyde dehydrogenase and alcohol dehydrogenase; PTA: phosphate acetyltransferase; ACK: acetate kinase. The red crosses indicate inactivated pathways in *L. lactis* CS4363 and CS436-F2. The light green question mark indicates possible pathway for pyruvate formation in *L. lactis* CS4363 and CS436-F2.

temperature of the column oven was set to 60 °C. Glucose, acetoin and 2,3-butanediol were quantified using an RI detector, while pyruvate, lactate and formate were read out from the UV detector at the wavelength of 210 nm. For the detection of ferricyanide, the absorbance of the supernatant was measured at 420 nm (Gu et al., 2023; Lai et al., 2016). For calibration, 7 concentrations of ferricyanide were used and then the formula was obtained:

$$Ferricyanide[mM] = 1.11 \times OD_{420}$$

The samples for the above analytics were collected and centrifuged at 17,000 *g*, 4 °C for 10 mins to remove the cell pellets. The supernatant was stored at −20 °C until analyzed.

2.5. Calculations

The optical cell density OD_{600} was converted into the cell dry weight (CDW) according to the following formula (Lan et al., 2006):

$$CDW[g/L] = 0.37 \times OD_{600}$$

The yield coefficients (Y) of quantified products were determined as the slope of plots of mmol product versus mmol glucose consumed (see Supplementary Materials).

The carbon balance (CB) was calculated according to the formula below:

$$CB(\%) = \sum_i (m_i \times n_i)_t / \sum_i (m_i \times n_i)_{t_0} \times 100$$

where m_i is the absolute quantity [mmol] of specific product *i* at specific time *t*; n_i is the carbon atom number of this product *i*; t_0 is the initial 0 h of inoculation. Due to the low production rate, formed CO_2 was estimated based on the stoichiometric coefficients from the respective metabolic pathway (Fig. 1) (Yu et al., 2018). The biomass formula was assumed to be $CH_{1.82}O_{0.54}N_{0.198}$ (Novák and Loubiere, 2000).

The electron balance (EB) was calculated according to the formula below:

$$EB(\%) = [\sum_i (m_i \times n_i \times \gamma)_t + e_{anode-t}] / [\sum_i (m_i \times n_i \times \gamma)_{t_0} + e_{anode-t_0}] \times 100$$

where $e_{anode\text{-}t}$ is the quantity [mmol] of electrons collected on the anode at specific time *t*; $e_{anode\text{-}t0}$ is the quantity [mmol] of electrons collected on the anode at initial 0 h. The degree of reduction (γ) of the respective chemical with the elemental composition $C_aH_bO_cN_d$ was calculated based on the formula (Stephanopoulos et al., 1998):

$$\gamma = \frac{a \times 4 + b \times 1 + c \times (-2) + d \times (-3)}{a}$$

The total turnover number (TTN) of ferricyanide was calculated based on the formula (Gemünde et al., 2023):

$$TTN = e_{anode-t} / (m_{mediator} \times z_{mediator})$$

where $m_{mediator}$ is the absolute quantity of mediator ferricyanide in the system; $z_{mediator}$ is the number of electrons that can be transferred by mediator in one turnover, for ferricyanide, it is equal to 1.

3. Results and discussion

3.1. SALN is a compatible medium for BES of L. Lactis

M17 medium with glucose is widely used for culturing *L. lactis* (Terzaghi and Sandine, 1975). However, an unknown oxidation event, at 0.36 V, occurred during a cyclic voltammetry testing of the blank medium (see Supplementary Materials). An unknown component in the GM17 medium was irreversibly oxidized at an onset potential of ca. + 0.04 V. This was not observed in previous experiments, and it may be due to batch to batch variation in M17 broth. This made GM17 medium incompatible with the planned electrochemical testing.

Due to this, the GM17 medium was exchanged with the chemically defined SALN medium (Solem et al., 2007). Riboflavin has been shown

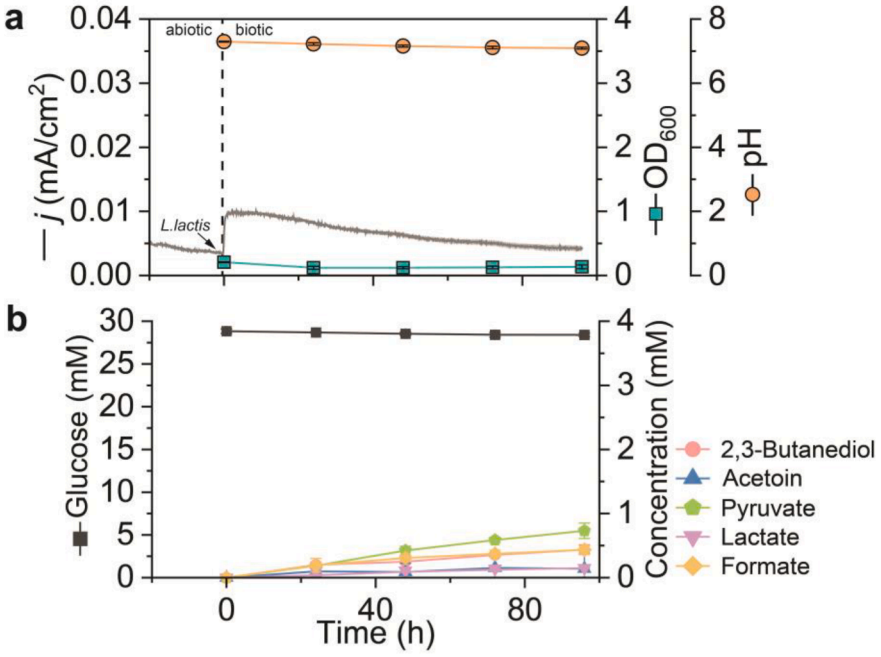

Fig. 2. Electrochemical activity and anoxic glucose metabolism of *L. lactis* CS4363 driven by BES. Working electrode potential was set up at 0.5 V versus Ag/AgCl. **(a)** Current density (*j*), pH and OD_{600}. **(b)** Glucose consumption and metabolic products. The presented data are the mean and standard deviations from biological replicates (N = 3).

to serve as a redox shuttle with a redox peak around −0.4 V vs. Ag/AgCl, and was left out as previously suggested (Masuda et al., 2010). The redox background noise observed for M17 was not seen for the SALN medium (see Supplementary Materials).

3.2. Hampered glucose metabolism in the BES without the exogenous mediator

L. lactis CS4363 is partly blocked in NAD^+ regeneration, and is unable to grow under strictly anaerobic conditions without alternative electron acceptors. *L. lactis* CS4363 has been shown to be able to use the endogenous mediator 2-amino-3-carboxy-1,4-naphthoquinone (ACNQ) to transfer electrons to electron acceptors (Gu et al., 2023). Considering that *L. lactis* is an electroactive bacterium, the anode here was used as the final electron acceptor for *L. lactis* CS4363 in the BES.

First it was tested whether CS4363 could grow in the BES without any mediators added. As shown in Fig. 2**a**, the max current density attained was quite low (0.0010 ± 0.0003 mA/cm^2) and only little glucose was consumed (0.43 ± 0.12 mM) over 90 h after inoculation. A slight drop in pH (0.20 ± 0.02) was observed, which indicated that *L. lactis* CS4363 can transfer electrons and protons out of the cells by using an endogenous mediator, similar to the observation in the previous research when using ferricyanide (Gu et al., 2023). However, the quantity of electrons transferred to the anode was only 0.723 ± 0.013 mmol, which was not sufficient to support the growth of CS4363. Pyruvate (0.73 ± 0.12 mM), 2,3-butanediol (0.44 ± 0.08 mM) and formate (0.44 ± 0.06 mM) were main products, and small amounts of lactate and acetoin could also be detected **(**Fig. 2**b)**. Although the genes encoding the three known lactate dehydrogenases (*ldh, ldhX, ldhB*) had been knocked out in CS4363 (Solem et al., 2013), a residual lactate dehydrogenase activity giving rise to lactate was apparently present. This could be due to some unannotated gene coding for an enzyme with lactate dehydrogenase activity, which has not been reported previously. In *L. lactis*, the pyruvate dehydrogenase complex (PDHc) is usually not active under anaerobic conditions (Snoep et al., 1993), whereas the pyruvate-formate lyase (PFL) is functional and responsible for decarboxylation of pyruvate to acetyl-CoA (Cocaign-Bousquet et al., 2002).

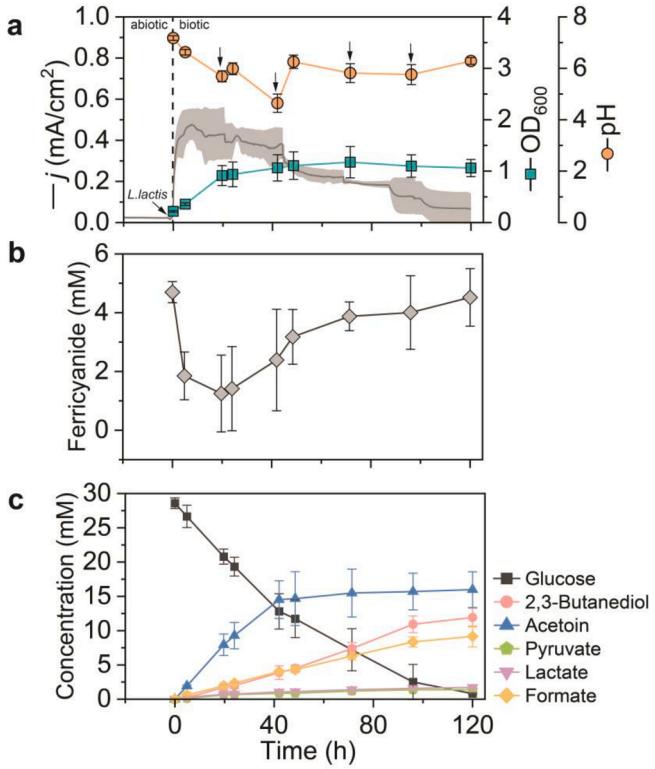

Fig. 3. Electrochemical activity and anoxic glucose metabolism of *L. lactis* CS4363 driven by BES with 5 mM ferricyanide as mediator. Working electrode potential was set up at 0.5 V versus Ag/AgCl. **(a)** Current density (*j*), pH and OD_{600}. **(b)** The concentration of potassium ferricyanide varies with time. **(c)** Glucose consumption and metabolic products. The arrows in (a) indicate the addition of 1 M KOH for adjustment of pH. The presented data are the mean and standard deviations from biological replicates (N = 3).

Table 1
Key progress parameters of glucose metabolism of CS4363 and CS4363-F2.

Strain	CS4363	CS4363-F2
Glucose consumed time in BES	120 h	24 h
CB (%)	112.32 ± 2.20	104.09 ± 2.08
EB (%)	114.48 ± 2.75	105.73 ± 2.36
μ_{max} (h^{-1})	0.068 ± 0.010	0.316 ± 0.038
$C_{x,max}$ (g_{CDW}/L)	0.44 ± 0.11	0.59 ± 0.08
Yields($mol_{product}$/$mol_{glucose}$)		
$Y_{2,3\text{-butanediol}}$	0.352 ± 0.022	0.801 ± 0.020
$Y_{acetoin}$	0.503 ± 0.048	0.040 ± 0.010
$Y_{pyruvate}$	0.041 ± 0.003	0.012 ± 0.003
$Y_{formate}$	0.263 ± 0.014	0.118 ± 0.017
$Y_{lactate}$	0.047 ± 0.002	0.060 ± 0.005
$Y_{electrons}$	3.234 ± 0.076	2.083 ± 0.070
Rates(mmol/g_{CDW}/h)		
$r_{glucose}$	2.246 ± 0.460	9.603 ± 2.228
$r_{2,3\text{-butanediol}}$	0.457 ± 0.069	5.714 ± 0.970
$r_{acetoin}$	2.024 ± 0.322	0.501 ± 0.156
$r_{pyruvate}$	0.149 ± 0.028	0.528 ± 0.070
$r_{formate}$	0.421 ± 0.125	0.776 ± 0.300
$r_{lactate}$	0.155 ± 0.041	0.784 ± 0.168
$r_{electrons}$	6.979 ± 1.096	11.471 ± 1.711

The detailed calculation of yield by linear fitting and rates of glucose consumption and other products formation were determined (see supplementary materials).

CS4363, however, normally does not accumulate formate, due to lack of phosphotransacetylase (PTA) and alcohol dehydrogenase (ADHE) activities (Gu et al., 2023), and thus it was unexpected that formate could be formed as there is no apparent sink for the acetyl-CoA generated.

3.3. Ferricyanide could facilitate transfer of electrons from CS4363 to an anode and thereby support its growth

In BES, electron transfer from microorganisms to the electrode is a major bottleneck (Vielstich et al., 2003). Since the observed interaction of CS4363 with the anode was very limited without an added mediator, 5 mM ferricyanide was added to the growth medium. A ferricyanide concentration that was 10 times lower than that used in the previous study (Gu et al., 2023) was chosen, since ferricyanide can be regenerated at the anode (Gemünde et al., 2023; Lai et al., 2016; Sun et al., 2023). To prevent accumulation of toxic HCN at low pH (Husmann et al., 2020), pH was maintained >= 6 by manually adding KOH when necessary.

The presence of 5 mM ferricyanide had a great impact, and the current density increased to a maximum of 0.47 ± 0.08 mA/cm^2 **(**Fig. 3**a)** and the electron formation rate was 6.979 ± 1.096 mmol/g_{CDW}/h (Table 1**)**. However, when KOH was added to maintain pH, a decrease in current was observed, possibly due to an excessive inward movement of K^+ that affected the transport of the endogenous mediator ACNQ, which further affected electron transfer from ACNQ to ferricyanide. Further work is needed to verify this hypothesis. Under these conditions, the final cell density (OD_{600}) of CS4363 reached 1.06 ± 0.17 in 120 h. After inoculation, the ferricyanide concentration dropped quickly to below 2 mM, due to its reduction by CS4363. After growth and current density had slowed down, ferricyanide gradually was fully re-oxidized by the anode **(**Fig. 3**b)**. Using the formula proposed by Gemünde et al. (Gemünde et al., 2023), for calculating the total turnover number (TTN) of ferricyanide in AEF, a TTN of 18.88 ± 0.59 was determined for CS4363, which demonstrates the reversibility of the ferricyanide redox reaction during the cultivation period. CB and EB of CS4363 exceeded 100% slightly **(**Table 1**)**. There could be two possible explanations for this: 1) the inoculated cells contained some intracellular glucose from the seed culture medium, while the measured initial glucose was only extracellular; 2) the carbon in the product came from other pathways, e.g. amino acid catabolism, which can also result in pyruvate which can be transformed into downstream metabolites (Le Bars and Yvon, 2008).

The 0.5% glucose initially present was fully depleted in 120 h and the glucose consumption rate was 2.246 ± 0.460 mmol/g_{CDW}/h **(**Table 1**)**, giving rise to a mixture of mainly acetoin, 2,3-butanediol, and formate. Small amounts of lactate and pyruvate were formed as well. Acetoin was the dominant metabolic product, which is consistent with the previous findings where ferricyanide was used as the final electron acceptor (Gu et al., 2023), and the yield for acetoin was 0.503 ± 0.048 $mol_{product}$/

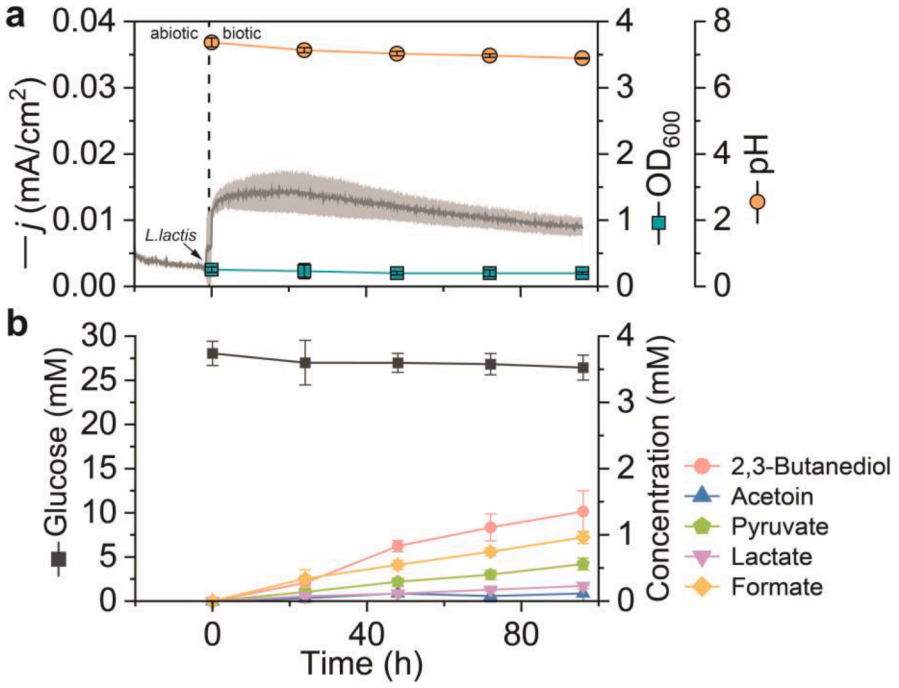

Fig. 4. Electrochemical activity and anoxic glucose metabolism of mutant *L. lactis* CS4363-F2 driven by BES. Working electrode potential was set up at 0.5 V versus Ag/AgCl. **(a)** Current density (*j*), pH and OD_{600}. **(b)** Glucose consumption and metabolic products. The presented data are the mean and standard deviations from biological replicates (N = 3).

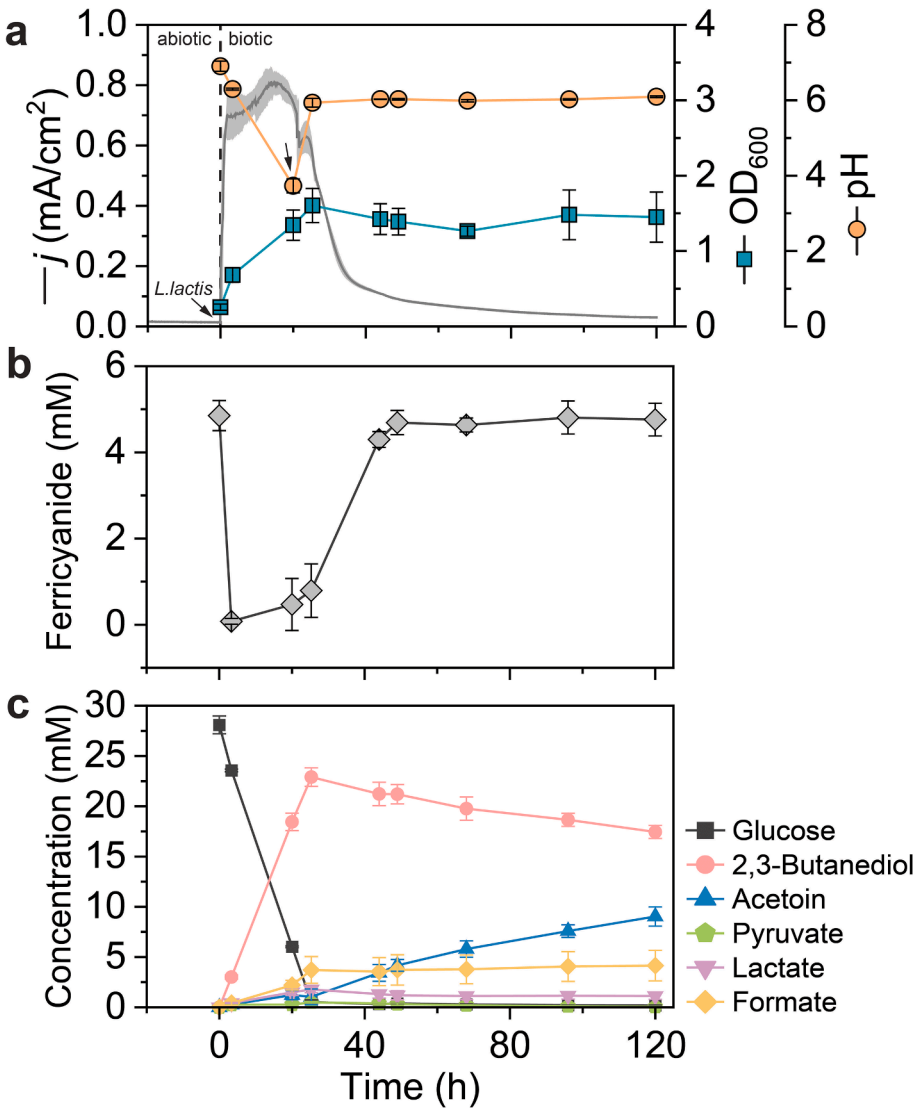

Fig. 5. Electrochemical activity and anoxic glucose metabolism of mutant *L. lactis* CS4363-F2 driven by BES with 5 mM ferricyanide as mediator. Working electrode potential was set up at 0.5 V versus Ag/AgCl. **(a)** Current density (*j*), pH and OD_{600}. **(b)** The concentration of potassium ferricyanide varies with time. **(c)** Glucose consumption and metabolic products. The arrow in (a) indicates the addition of 1 M KOH for adjustment of pH. The presented data are the mean and standard deviations from biological replicates (N = 3).

$mol_{glucose}$ (Fig. 3**c**). The final concentration of acetoin and 2,3-butanediol reached 15.98 ± 2.60 mM and 11.91 ± 1.35 mM, respectively. The product distribution in the presence of ferricyanide was different from when it was absent, e.g. pyruvate no longer accumulated in significant amounts, and was converted to downstream metabolites. Overall, the NADH generated in glycolysis could be re-oxidized by the anode in the presence of ferricyanide as electron mediator, thereby establishing redox balance, which is a basic requirement for living cells (Chen et al., 2014). CS4363 was found to donate more electrons in the BES setup (666.87 ± 16.25 mM) as compared to when grown under non-BES conditions (345.66 ± 13.19 mM) (Gu et al., 2023), which could also been seen from the glucose consumption. However, the growth rate in BES was 0.068 ± 0.010 h^{-1} (Table 1), i.e. significantly lower than non-BES condition (μ_{max} = 0.419 ± 0.006 h^{-1}) (Gu et al., 2023). The faster growth under non-BES conditions can be explained by differences in medium and cultivation conditions since non-BES experiments were carried out in rich M17 medium with higher nutritional content and the growth condition was not completely anaerobic. Besides, it seems that electron transfer to the electrode limits growth in the current BES setup. There could be two explanations for this. Either ferricyanide was regenerated too slowly by the anode, or perhaps the reduced ferricyanide concentration hampered electron transfer between the cell and ferricyanide. The latter phenomenon appears to be the case as next results demonstrate.

3.4. Enhancing capacity for ferricyanide respiration enhances performance in the BES

In the previous research, CS4363 was adaptively evolved to enhance its capacity for EET with ferricyanide, and one of the mutants obtained was CS4363-F2. CS4363-F2 was further characterized by using the same BES setup. In the absence of ferricyanide, CS4363-F2 grew poorly and only metabolized little glucose (Fig. 4**a**), although glucose consumption increased approximately by a factor 3 to 1.61 ± 0.35 mM and the pH drop was more significant (0.48 ± 0.15) than for CS4363. Another difference between the two strains was that the dominant fermentation product was 2,3-butanediol (1.35 ± 0.31 mM) rather than pyruvate (Fig. 4**b**), findings that are partly consistent with previous results (Gu et al., 2023).

In the presence of 5 mM ferricyanide the situation, however, changed

radically. As shown in Fig. 5**a**, the current density increased to 0.81 ± 0.05 mA/cm^2 (equivalent to 20.25 mA), which was about twice that observed for CS4363, and record high when compared to current densities reported by others (see Supplementary Materials). Besides, CS4363-F2 also displayed enhanced electron formation rate (11.471 ± 1.711 mmol/g_{CDW}/h) in comparison to CS4363, which demonstrated that this mutant had enhanced electron transfer ability.

The time needed for CS4363-F2 to reach the stationary phase was shortened to 24 h, half of the time needed for CS4363. The final OD_{600} reached 1.45 ± 0.33 **(**Fig. 5**a)** and the growth rate increased to 0.316 ± 0.038 h^{-1} **(**Table 1**)**. The maximum CDW also increased to 0.59 ± 0.08 g_{CDW}/L, while the maximum CDW of CS4363 was 0.44 ± 0.11 g_{CDW}/L. CS4363-F2, almost immediately, depleted ferricyanide within 3 h after inoculation, which illustrates the superior ability of CS4363-F2 to respire with ferricyanide **(**Fig. 5**b)**. Thus, enhancing the capacity for EET with ferricyanide, greatly enhanced ability for anodic electro-fermentation. After the cessation of growth after 24 h, the concentration of ferricyanide gradually increased to the initial 5 mM and was fully regenerated at 120 h **(**Fig. 5**b)**.

For CS4363-F2, the time needed to deplete glucose was less than 24 h, as compared to 120 h for CS4363. The glucose consumption rate was increased to 9.603 ± 2.228 mmol/g_{CDW}/h. In contrast to what was observed for CS4363, CS4363-F2 mostly converted glucose into 2,3-butanediol (22.90 ± 0.92 mM) and only small amounts of acetoin were generated, 1.04 ± 0.78 mM at 24 h **(**Fig. 5**c)**. The amount of 2,3-butanediol generated was similar to that under non-BES condition (Gu et al., 2023), while only half of the biomass had accumulated. In CS4363-F2, the *butBA* operon, encoding the 2,3-butanediol dehydrogenase, has been massively amplified (Gu et al., 2023), and this had an impact on the yield of 2,3-butanediol which reached 0.801 ± 0.020 $mol_{product}/mol_{glucose}$, which was around twice as much as formed by CS4363 (0.352 ± 0.022 $mol_{product}/mol_{glucose}$). In addition, 2,3-butanediol was formed 12.5 times faster by CS4363-F2 (5.714 ± 0.970 mmol/g_{CDW}/h) than by CS4363 (0.457 ± 0.069 mmol/g_{CDW}/h). For acetoin, the yield for CS4363 reached 0.503 ± 0.048 $mol_{product}/mol_{glucose}$, whereas the yield for CS4363-F2 only was 0.040 ± 0.010 $mol_{product}/mol_{glucose}$ **(**Table 1**)**.

After 24 h, growth ceased due to glucose depletion. An interesting finding was that after cells had entered the stationary phase, 2,3-butanediol was gradually reduced into acetoin. Thus the anode facilitated biotransformation by non-growing cells, where the 2,3-butanediol dehydrogenase functioned in the reverse orientation, generating acetoin and NADH, where the latter was oxidized back to NAD$^+$ by EET to ferricyanide, thereby funneling additional electrons to the anode.

4. Conclusion

This is the first study describing the potential of anode-assisted electro-fermentation of growing *L. lactis*. In an anodic BES setup, growth profile and product composition of NAD$^+$ regeneration-blocked *L. lactis* strains vary with electron mediator ferricyanide presence or not. The ALE strain CS4363-F2 displays remarkable performance in a BES setup, achieving record high current densities not previously observed, indicating it is possible to enhance the capacity for AEF by adapting the microorganism to respire better with ferricyanide. CS4363-F2 is also proved to be an efficient cell factory for producing the bulk chemical 2,3-butanediol in BES setup.

CRediT authorship contribution statement

Liuyan Gu: Conceptualization, Formal analysis, Investigation, Visualization, Validation, Writing – original draft, Writing – review & editing. **Xinxin Xiao:** Writing – review & editing, Resources, Investigation. **Sang Yup Lee:** Writing – review & editing, Supervision, Funding acquisition. **Bin Lai:** Writing – review & editing, Conceptualization, Formal analysis, Investigation, Resources, Supervision, Methodology. **Christian Solem:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2023.129813.

References

Cesselin, B., Derré-Bobillot, A., Fernandez, A., Lamberet, G., Lechardeur, D., Yamamoto, Y., Pedersen, M.B., Garrigues, C., Gruss, A., Gaudu, P., 2011. Responses of lactic acid bacteria to oxidative stress. In: Tsakalidou, E., Papadimitriou, K. (Eds.), Stress Responses of Lactic Acid Bacteria. Springer, US, Boston, MA, pp. 111–127. https://doi.org/10.1007/978-0-387-92771-8_6.

Chen, X., Li, S., Liu, L., 2014. Engineering redox balance through cofactor systems. Trends in Biotechnology 32, 337–343. https://doi.org/10.1016/j.tibtech.2014.04.003.

Cocaign-Bousquet, M., Even, S., Lindley, N.D., Loubière, P., 2002. Anaerobic sugar catabolism in *Lactococcus lactis*: genetic regulation and enzyme control over pathway flux. Applied Microbiology and Biotechnology 60, 24–32. https://doi.org/10.1007/s00253-002-1065-x.

Delvigne, F., Lecomte, J., 2010. Foam formation and control in bioreactors. In: Encyclopedia of Industrial Biotechnology. John Wiley & Sons Ltd, pp. 1–13. https://doi.org/10.1002/9780470054581.eib326.

Doran, P.M., 1995. 9 - Mass Transfer. In: Doran, P.M. (Ed.), Bioprocess Engineering Principles. Academic Press, London, pp. 190–217. https://doi.org/10.1016/B978-012220855-3/50009-2.

Duwat, P., Sourice, S., Cesselin, B., Lamberet, G., Vido, K., Gaudu, P., Le Loir, Y., Violet, F., Loubière, P., Gruss, A., 2001. Respiration capacity of the fermenting bacterium *Lactococcus lactis* and its positive effects on growth and survival. Journal of Bacteriology 183, 4509–4516. https://doi.org/10.1128/JB.183.15.4509-4516.2001.

Garcia-Ochoa, F., Gomez, E., 2009. Bioreactor scale-up and oxygen transfer rate in microbial processes: An overview. Biotechnology Advances 27, 153–176. https://doi.org/10.1016/j.biotechadv.2008.10.006.

Gemünde, A., Gail, J., Holtmann, D., 2023. Anodic respiration of *Vibrio natriegens* in a bioelectrochemical system. ChemSusChem 1–8. https://doi.org/10.1002/cssc.202300181.

Gibson, B.R., Lawrence, S.J., Boulton, C.A., Box, W.G., Graham, N.S., Linforth, R.S.T., Smart, K.A., 2008. The oxidative stress response of a lager brewing yeast strain during industrial propagation and fermentation. FEMS Yeast Research 8, 574–585. https://doi.org/10.1111/j.1567-1364.2008.00371.x.

Gong, Z., Yu, H., Zhang, J., Li, F., Song, H., 2020. Microbial electro-fermentation for synthesis of chemicals and biofuels driven by bi-directional extracellular electron transfer. Synthetic and Systems Biotechnology 5, 304–313. https://doi.org/10.1016/j.synbio.2020.08.004.

Gu, L., Xiao, X., Zhao, G., Kempen, P., Zhao, S., Liu, J., Lee, S.Y., Solem, C., 2023. Rewiring the respiratory pathway of *Lactococcus lactis* to enhance extracellular electron transfer. Microbial Biotechnology 16, 1277–1292. https://doi.org/10.1111/1751-7915.14229.

Husmann, S., Zarbin, A.J.G., Dryfe, R.A.W., 2020. High-performance aqueous rechargeable potassium batteries prepared via interfacial synthesis of a Prussian

blue-carbon nanotube composite. Electrochimica Acta 349, 136243. https://doi.org/10.1016/j.electacta.2020.136243.
Jensen, P.R., Hammer, K., 1993. Minimal requirements for exponential growth of *Lactococcus lactis*. Applied and Environmental Microbiology 59, 4363–4366. https://doi.org/10.1128/aem.59.12.4363-4366.1993.
Kim, C., Kim, M.Y., Michie, I., Jeon, B.-H., Premier, G.C., Park, S., Kim, J.R., 2017. Anodic electro-fermentation of 3-hydroxypropionic acid from glycerol by recombinant *Klebsiella pneumoniae* L17 in a bioelectrochemical system. Biotechnology for Biofuels 10, 199. https://doi.org/10.1186/s13068-017-0886-x.
Kirsop, B.H., 1974. Oxygen in brewery fermentation. Journal of the Institute of Brewing 80, 252–259. https://doi.org/10.1002/j.2050-0416.1974.tb03614.x.
Koebmann, B., Blank, L.M., Solem, C., Petranovic, D., Nielsen, L.K., Jensen, P.R., 2008. Increased biomass yield of *Lactococcus lactis* during energetically limited growth and respiratory conditions. Biotechnology and Applied Biochemistry 50, 25–33. https://doi.org/10.1042/BA20070132.
Lai, B., Yu, S., Bernhardt, P.V., Rabaey, K., Virdis, B., Krömer, J.O., 2016. Anoxic metabolism and biochemical production in *Pseudomonas putida* F1 driven by a bioelectrochemical system. Biotechnology for Biofuels 9, 39. https://doi.org/10.1186/s13068-016-0452-y.
Lai, B., Nguyen, A.V., Krömer, J.O., 2019. Characterizing the anoxic phenotype of *Pseudomonas putida* using a bioelectrochemical system. Methods Protocol 2, 26. https://doi.org/10.3390/mps2020026.
Lan, C.Q., Oddone, G., Mills, D.A., Block, D.E., 2006. Kinetics of *Lactococcus lactis* growth and metabolite formation under aerobic and anaerobic conditions in the presence or absence of hemin. Biotechnology and Bioengineering 95, 1070–1080. https://doi.org/10.1002/bit.21070.
Le Bars, D., Yvon, M., 2008. Formation of diacetyl and acetoin by *Lactococcus lactis* via aspartate catabolism. Journal of Applied Microbiology 104, 171–177. https://doi.org/10.1111/j.1365-2672.2007.03539.x.
Lechardeur, D., Cesselin, B., Fernandez, A., Lamberet, G., Garrigues, C., Pedersen, M., Gaudu, P., Gruss, A., 2011. Using heme as an energy boost for lactic acid bacteria. Current Opinion in Biotechnology 22, 143–149. https://doi.org/10.1016/j.copbio.2010.12.001.
Li, Q., Bai, Z., O'Donnell, A., Harvey, L.M., Hoskisson, P.A., McNeil, B., 2011. Oxidative stress in fungal fermentation processes: the roles of alternative respiration. Biotechnology Letters 33, 457–467. https://doi.org/10.1007/s10529-010-0471-x.
Liu, J.-M., Chen, L., Dorau, R., Lillevang, S.K., Jensen, P.R., Solem, C., 2020. From waste to taste—efficient production of the butter aroma compound acetoin from low-value dairy side streams using a natural (nonengineered) *Lactococcus lactis* dairy isolate. Journal of Agricultural and Food Chemistry 68, 5891–5899. https://doi.org/10.1021/acs.jafc.0c00882.
Lopez de Felipe, F., Kleerebezem, M., de Vos, W.M., Hugenholtz, J., 1998. Cofactor engineering: a novel approach to metabolic engineering in *Lactococcus lactis* by controlled expression of NADH oxidase. Journal of Bacteriology 180, 3804–3808. https://doi.org/10.1128/jb.180.15.3804-3808.1998.
Lv, P.J., Qiang, S., Liu, L., Hu, C.Y., Meng, Y.H., 2020. Dissolved-oxygen feedback control fermentation for enhancing β-carotene in engineered *Yarrowia lipolytica*. Scientific Reports 10, 17114. https://doi.org/10.1038/s41598-020-74074-0.
Masuda, M., Freguia, S., Wang, Y.-F., Tsujimura, S., Kano, K., 2010. Flavins contained in yeast extract are exploited for anodic electron transfer by *Lactococcus lactis*. Bioelectrochemistry 78, 173–175. https://doi.org/10.1016/j.bioelechem.2009.08.004.
Moscoviz, R., Toledo-Alarcón, J., Trably, E., Bernet, N., 2016. Electro-fermentation: How to drive fermentation using electrochemical systems. Trends in Biotechnology 34, 856–865. https://doi.org/10.1016/j.tibtech.2016.04.009.
Nordkvist, M., Jensen, N.B.S., Villadsen, J., 2003. Glucose metabolism in *Lactococcus lactis* MG1363 under different aeration conditions: requirement of acetate to sustain growth under microaerobic conditions. Applied and Environmental Microbiology 69, 3462–3468. https://doi.org/10.1128/AEM.69.6.3462-3468.2003.
Novák, L., Loubiere, P., 2000. The metabolic network of *Lactococcus lactis*: distribution of 14C-Labeled substrates between catabolic and anabolic pathways. Journal of Bacteriology 182, 1136–1143. https://doi.org/10.1128/jb.182.4.1136-1143.2000.
Pedersen, M.B., Gaudu, P., Lechardeur, D., Petit, M.-A., Gruss, A., 2012. Aerobic respiration metabolism in lactic acid bacteria and uses in biotechnology. Annual Review of Food Science and Technology 3, 37–58. https://doi.org/10.1146/annurev-food-022811-101255.
Rezaïki, L., Cesselin, B., Yamamoto, Y., Vido, K., Van West, E., Gaudu, P., Gruss, A., 2004. Respiration metabolism reduces oxidative and acid stress to improve long-term survival of *Lactococcus lactis*. Molecular Microbiology 53, 1331–1342. https://doi.org/10.1111/j.1365-2958.2004.04217.x.
Sano, A., Takatera, M., Kawai, M., Ichinose, R., Yamasaki-Yashiki, S., Katakura, Y., 2020. Suppression of lactate production by aerobic fed-batch cultures of *Lactococcus lactis*. Journal of Bioscience and Bioengineering 130, 402–408. https://doi.org/10.1016/j.jbiosc.2020.06.004.
Snoep, J.L., de Graef, M.R., Westphal, A.H., de Kok, A., Teixeira, J., de Mattos, M., Neijssel, O.M., 1993. Differences in sensitivity to NADH of purified pyruvate dehydrogenase complexes of *Enterococcus faecalis*, *Lactococcus lactis*, *Azotobacter vinelandii* and *Escherichia coli*: Implications for their activity in vivo. FEMS Microbiology Letters 114, 279–283. https://doi.org/10.1111/j.1574-6968.1993.tb06586.x.
Solem, C., Koebmann, B., Yang, F., Jensen, P.R., 2007. The las enzymes control pyruvate metabolism in *Lactococcus lactis* during growth on maltose. Journal of Bacteriology 189, 6727–6730. https://doi.org/10.1128/jb.00902-07.
Solem, C., Dehli, T., Jensen, P.R., 2013. Rewiring *Lactococcus lactis* for ethanol production. Applied and Environmental Microbiology 79, 2512–2518. https://doi.org/10.1128/AEM.03623-12.
Stephanopoulos, G., Aristidou, A.A., Nielsen, J., 1998. Metabolic Engineering: principles and methodologies. Elsevier.
Sun, Y., Kokko, M., Vassilev, I., 2023. Anode-assisted electro-fermentation with *Bacillus subtilis* under oxygen-limited conditions. Biotechnology for Biofuels and Bioproducts 16, 6. https://doi.org/10.1186/s13068-022-02253-4.
Suttikul, S., Charalampopoulos, D., Chatzifragkou, A., 2023. Biotechnological production of optically pure 2,3-butanediol by *Bacillus subtilis* based on dissolved oxygen control strategy. Fermentation 9, 15. https://doi.org/10.3390/fermentation9010015.
Sybesma, W., Burgess, C., Starrenburg, M., van Sinderen, D., Hugenholtz, J., 2004. Multivitamin production in *Lactococcus lactis* using metabolic engineering. Metabolic Engineering 6, 109–115. https://doi.org/10.1016/j.ymben.2003.11.002.
Terzaghi, B.E., Sandine, W.E., 1975. Improved medium for lactic streptococci and their bacteriophages. Applied and Environmental Microbiology 29, 807–813. https://doi.org/10.1128/am.29.6.807-813.1975.
Vassilev, I., Gießelmann, G., Schwechheimer, S.K., Wittmann, C., Virdis, B., Krömer, J.O., 2018. Anodic electro-fermentation: Anaerobic production of L-Lysine by recombinant *Corynebacterium glutamicum*. Biotechnology and Bioengineering 115, 1499–1508. https://doi.org/10.1002/bit.26562.
Vassilev, I., Averesch, N.J.H., Ledezma, P., Kokko, M., 2021. Anodic electro-fermentation: Empowering anaerobic production processes via anodic respiration. Biotechnology Advances 48, 107728. https://doi.org/10.1016/j.biotechadv.2021.107728.
Vielstich, W., Lamm, A., Gasteiger, A., H.,, 2003. Handbook of fuel cells: fundamentals. John Wiley & Sons technology, applications, 4 volume set.
Virdis, B., D. Hoelzle, R., Marchetti, A., Boto, S.T., Rosenbaum, M.A., Blasco-Gómez, R., Puig, S., Freguia, S., Villano, M., 2022. Electro-fermentation: Sustainable bioproductions steered by electricity. Biotechnology Advances 59, 107950. https://doi.org/10.1016/j.biotechadv.2022.107950.
Yu, S., Lai, B., Plan, M.R., Hodson, M.P., Lestari, E.A., Song, H., Krömer, J.O., 2018. Improved performance of *Pseudomonas putida* in a bioelectrochemical system through overexpression of periplasmic glucose dehydrogenase. Biotechnology and Bioengineering 115, 145–155. https://doi.org/10.1002/bit.26433.
Zhao, G., Liu, J., Zhao, J., Dorau, R., Jensen, P.R., Solem, C., 2021. Efficient production of nisin A from low-value dairy side streams using a nonengineered dairy *Lactococcus lactis* strain with low lactate dehydrogenase activity. Journal of Agricultural and Food Chemistry 69, 2826–2835. https://doi.org/10.1021/acs.jafc.0c07816.