

7 Dynamics of pyruvate metabolism in *Lactococcus lactis*

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Introduction

The lactic acid bacterium *Lactococcus lactis* is commonly used in the dairy industry for the manufacture of fermented milk and cheese. This is mainly due to the ability of this organism to rapidly convert the sugars present in milk to lactic acid, whereby a preservative effect is obtained. *L. lactis* is generally considered homofermentative, i.e., lactic acid is produced as the major end product, and when grown on a rapidly fermentable sugar such as glucose, more than 90% of the glucose consumed is recovered in lactic acid. However, when grown on a less favourable sugar such as galactose, a significant fraction of the pyruvate pool is diverted from lactic acid towards the mixed acid fermentation products formate, acetate and ethanol.

The primary metabolism of *L. lactis* is quite simple (Fig. 7.1), but the regulation of the metabolic shift from homolactic to mixed acid product formation is complex and has challenged researchers for decades. The glycolytic intermediates glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP) have been shown to allosterically inhibit the activity of the pyruvate formate-lyase (PFL) enzyme [8]. Furthermore, fructose-1,6-diphosphate (FDP) is a necessary activator of the lactate dehydrogenase (LDH) enzyme [4,9]. The intracellular concentrations of FDP, GAP and DHAP are high during growth in batch cultures on glucose [5], whereby LDH is activated and PFL is inhibited. In contrast, the levels of FDP, GAP and DHAP are low during growth on galactose [5], leading to alleviation of PFL inhibition and reduced activation of LDH. This model based on allosteric control is generally used to explain the metabolic shift, but it has recently been shown that the transcription of *pfl* gene, encoding PFL, is induced during growth on galactose

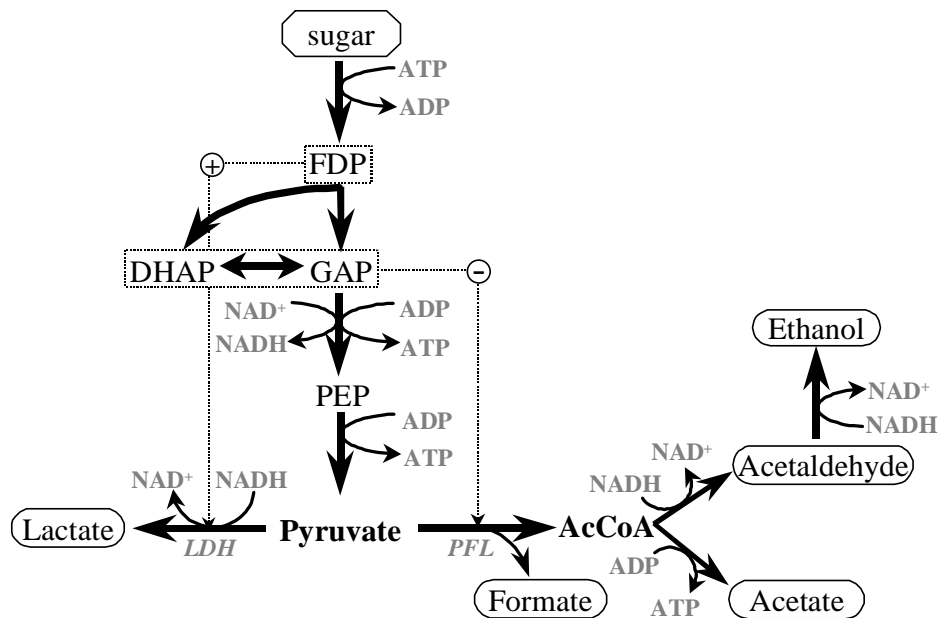


Fig. 7.1 Fermentative metabolism of *Lactococcus lactis*.

compared to glucose [1]. Furthermore, we have shown that the PFL enzyme level is 5-fold higher during growth on galactose compared to glucose [7], suggesting that regulated *pfl* gene expression plays an important role for the metabolic shift. To assess the relative significance of the different regulatory mechanisms, transient experiments are carried out with glucose as substrate. In a steady-state culture at low dilution rate, the glycolytic flux is low, presumably giving a product pattern similar to that observed in batch cultures on galactose, while growth at a high dilution rate should give the homolactic product formation known from batch cultivations on glucose. A steady-state culture at a low dilution rate was exposed to a sudden increase in dilution rate, and the dynamic response of the organism was monitored by measuring end products, intracellular metabolites and specific enzyme levels.

Materials and methods

Organism and culturing conditions *Lactococcus lactis* subsp. *cremoris* MG1363 was cultivated in a 2 L MBR Bioreactor. Cells were grown in defined MS10 medium [2] supplemented with 10 g/L of glucose. Anaerobic conditions were ensured by a constant flow of nitrogen through the headspace of the bioreactor. A constant pH of 6.6 was maintained by controlled addition of 5 M KOH. A steady-state was obtained at $D = 0.1 \text{ h}^{-1}$ by addition of 100 ml/h of feed to the bioreactor operating with a working volume of 1 L. The biomass concentration was monitored by mea-

suring the optical density at 600 nm (OD_{600}), and one unit of OD_{600} was shown to be equivalent to 0.354 (g cell dry weight)/L.

Measurement of extracellular metabolites For determination of extracellular metabolites, 1 ml sample was filtered through a 0.22 μ m pore size filter. Glucose, lactate, formate, acetate and ethanol were separated on an Aminex HPX-87H column (Biorad, CA) using 0.6 ml/min 5 mM H_2SO_4 as mobile phase. Glucose and ethanol were measured refractometrically using a Waters 410 Differential Refractometer Detector (Millipore Corp., MA). Lactate, formate and acetate were detected using a Waters 486 Tunable Absorbance Detector at 210 nm.

Measurement of intracellular metabolites Samples for measurement of intracellular metabolites were taken through a fast sampling valve into $-40^\circ C$ methanol to ensure rapid inactivation of the metabolism. Purification and detection of intracellular metabolites was done as described in [6].

Quantification of PFL and LDH enzyme levels A volume of 20–40 ml of culture was collected from the effluent of the bioreactor. Cells were isolated by centrifugation and stored at $-20^\circ C$ until analysis. Preparation of cell free extracts and the *in vitro* activity assay of LDH was done as described in [5]. The PFL protein level per mg total protein was measured by sandwich ELISA as described in [7].

Results and discussion

Transient behaviour of pyruvate metabolism in *L. lactis*

The regulation of the metabolic shift from mixed acid to homolactic product formation may take place by allosteric modulation of enzyme activities but also by altered levels of enzymes competing for pyruvate, i.e., LDH and PFL. Regulation by allosteric modulation is presumably rapidly imposed, whereas regulation by readjustment of enzyme levels is slower since it involves *de novo* synthesis and/or degradation of protein. To estimate the relative significance of these regulatory mechanisms, transient experiments are carried out. A steady-state was obtained in a chemostat operating at a constant dilution rate (D) of $0.1\ h^{-1}$. In Fig. 7.2, the data points for time $t \leq 0$ represent this steady-state. Mixed acid product formation was observed at $D = 0.1\ h^{-1}$, and PFL was the predominant enzyme with an *in vivo* activity of 5.8 mmol/(h·g cell dry weight) compared to 1.7 mmol/(h·g cell dry weight) for LDH. At time $t = 0$, the dilution rate was increased to $D = 0.5\ h^{-1}$. The sudden increase in dilution rate led to an initial washout of biomass ($x/x_0 = \exp[(0.1 - 0.5)t]$) and rapid accumulation of glucose.

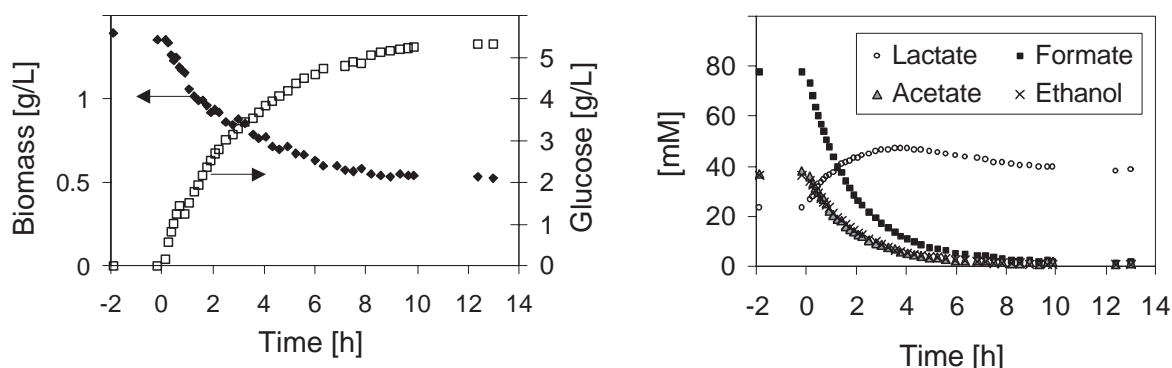


Fig. 7.2 Biomass and extracellular metabolites during the shift-up experiment. For time $t \leq 0$, a steady-state at $D = 0.1 \text{ h}^{-1}$ was maintained, and at $t = 0$ the dilution rate was increased to 0.5 h^{-1} .

This shows that *L. lactis* after being adapted to a low dilution rate, had an insufficient anabolic capacity to utilise the higher sugar level available at $D = 0.5 \text{ h}^{-1}$. The shift-up in dilution rate changed the end product formation dramatically. Strikingly, the concentration profiles for the mixed acid products followed the profiles for the washout of these products ($c/c_0 = \exp[-0.5t]$), demonstrating that there was virtually no PFL *in vivo* activity after the shift-up. The *in vivo* activity of LDH increased to 24 mmoles/(h·g cell dry weight) immediately after the shift-up (Time, $0 < t < 0.5 \text{ h}$), corresponding to a 14-fold increase compared to the previous steady-state level. The total flux through glycolysis consequently increased by a factor of more than 3 ($24/[5.8 + 1.7]$). The rapid quenching of PFL activity shows that allosteric modulation was the predominant regulatory mechanism. To verify this, intracellular metabolites and enzyme levels were measured as described below.

Regulation of the metabolic shift

The mechanisms responsible for the shift in pyruvate catabolism were analysed by measuring LDH and PFL enzyme levels and intracellular metabolites during the shift-up experiment.

The LDH enzyme level was measured as *in vitro* activities in protein extracts. The level of LDH increased during the transient from approximately 7 to 14 $\mu\text{moles} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ (Fig. 7.3), a moderate increase compared to the 14-fold increase of *in vivo* LDH activity observed immediately after the shift-up. This clearly shows that variations in the LDH enzyme level only played a minor role in the regulation of the shift. In contrast, the shift-up caused the intracellular environment to change towards conditions that were more favourable for the LDH enzyme. The stimulation of LDH might have been mediated by the enhanced level of the activator FDP that increased from 33 to 46 mM (Fig. 7.3). However, the FDP concen-

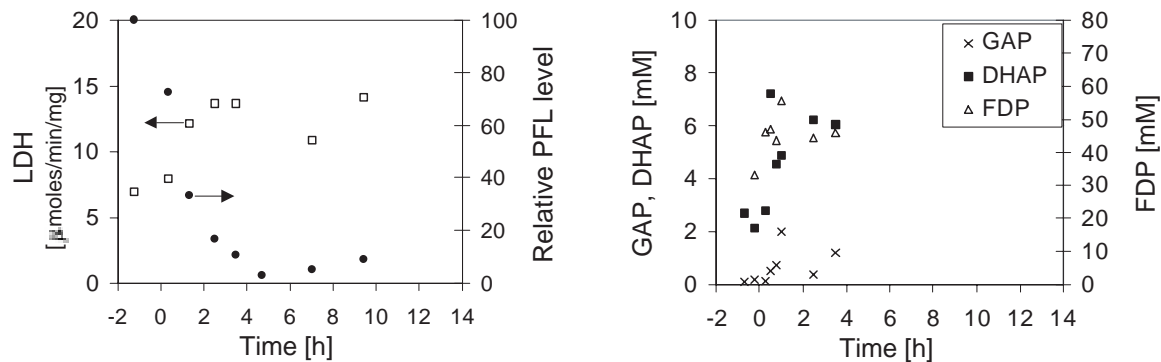


Fig. 7.3 LDH and PFL enzyme levels and intracellular metabolites during shift-up experiment. The PFL level per mg total protein was normalised with respect to the steady-state level of PFL (Time $t < 0$).

tration required for half-maximal activity of the LDH enzyme has been reported to be in the range of 1 mM [3], and it is therefore reasonable to assume that the enzyme was fully saturated with respect to FDP both before and after the shift. Hence, it is likely that LDH activity was enhanced by other means, e.g., by a higher intracellular concentration of pyruvate or a higher NADH /NAD⁺ ratio.

The PFL level per mg total protein was measured by ELISA, and the PFL levels shown in Fig. 7.3 were normalised with respect to the steady-state PFL level. The shift-up in dilution rate caused a remarkable decrease in the level of PFL, and towards the end of the experiment, the PFL level corresponded to only 5–10% of the steady-state level. The initial cells containing a high level of PFL were slowly washed out, while new cells only contained a low level of PFL that presumably was required to sustain the formation of acetyl-CoA needed for lipid synthesis. However, as mentioned above, the PFL *in vivo* activity was inhibited immediately after the shift-up, and the changed product formation can therefore not be ascribed to a lower PFL enzyme level. On the contrary, the strongly reduced PFL *in vivo* activity was caused by allosteric inhibition of PFL resulting from the higher intracellular concentrations of GAP and DHAP observed after the shift-up (Fig. 7.3). Thus, the adjustment of the PFL protein level may not have any significant role in the regulation of the metabolic shift under the conditions analysed. Currently experiments are carried out to analyse the dynamic response of the pyruvate metabolism in *L. lactis* under conditions, where both allosteric modulation and adjustment of enzyme levels contribute to the metabolic shift.

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