Metabolic flux in glucose/citrate co-fermentation by lactic acid bacteria as measured by isotopic ratio analysis

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Abstract

The flux of carbon into lactic acid, diacetyl and acetoin during the co-metabolism of glucose and citrate by Lactococcus lactis subsp. lactis biovar. diacetylactis has been determined using natural abundance isotopic ratio analysis. During fermentation in the conditions used (glucose, 27.8 mM; citric acid, 13.9 mM; initial pH 6.2–6.4, anaerobic) it is shown that approximately 65% of the carbon source used for the aroma compounds is derived from the carbohydrate. Equally, citrate contributes approximately 30% of the carbon recovered in lactic acid. Thus, there is no evidence for a metabolic separation of the catabolism of these two carbon sources. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

The co-fermentation of lactose and citrate in milk by Lactococcus bacteria leads to the accumulation of lactic acid, the major product, and a number of minor products important for organoleptic and textural qualities [1]. Lactic acid provides protection against spoilage by non-acidophilic organisms. For the organoleptic properties, the C4 compound diacetyl and, to a much lesser extent, its partially reduced form, acetoin, are responsible for the ‘buttery’ flavor notes. These two compounds accumulate in fermentations using Lactococcus lactis subsp. lactis biovar. diacetylactis provided with a carbohydrate source (lactose or glucose) and citrate.

Biosynthetically, lactic acid, diacetyl and acetoin are all derived from pyruvate (Fig. 1), lactic acid via the direct action of lactate dehydrogenase (LDH), diacetyl and acetoin from 2 mol of pyruvate via α-acetolactate [2]. Pyruvate is produced from both of the two main sources of fermentable carbon in culture medium, glucose and citrate. While the interrelationship of the fermentation of these two compounds is complex [3], fermentation at neutral or slightly alkaline pH initially uses glucose, citrate catabolism only becoming significant as the pH of the medium drops below 6.5. Thereafter, the pool of pyruvate can effectively be replenished from either carbon source. Potentially, glucose can provide two units of pyruvate per unit of substrate, citrate only one. Thus, in principle, both the carbohydrate and citrate are available for the biosynthesis of both lactic acid and the aromatic products, diacetyl and acetoin.

Several studies have shown that the co-metabolism of citrate with glucose radically changes the metabolite profile generated during anaerobic fermentation by lactic acid bacteria. A number of lines of evidence have been interpreted as indicating that lactic acid is principally derived from glucose but that diacetyl and acetoin are primary produced from citrate. Notably, (1) fermentations conducted in the absence of citrate accumulate negligible amounts of these aromatic compounds; (2) diacetyl and acetoin have been reported to accumulate late in the fermentation period, coincident with the period after citrate catabolism has started [4]; (3) aerobic fermentations favor diacetyl and acetoin accumulation, in accord with the oxidative metabolism of citrate [5].

Such evidence is, however, indirect. In a study using 13C NMR and specifically-labelled citrate fed to non-growing cultures of several strains of L. lactis subsp. lactis 19B, it was found that at pH 5.5, 50–70% of available citrate was converted to lactic acid [2]. Furthermore, in both non-
growing and growing cultures (initial pH 6.4), while citrate alone was substantially converted to acetoin, the co-metabolism of citrate with glucose led to up to 80% bioconversion of citrate to lactic acid [6]. These studies have several major drawbacks. First, metabolism in non-growing cells is unlikely to mimic that in exponentially growing cultures. Second, by using constant conditions, the evolution of citrate metabolism with pH was not followed. Third, in neither instance was the key flavor compound, diacetyl, detected.

In order to overcome such problems, the co-metabolism of glucose and citrate has been investigated using the small variation in natural $^{13}$C content between substrates derived from different biological sources. Due to the different kinetic isotope effects of the enzymes involved in carbon fixation between plants that exploit the C-3 and C-4 pathways [7], glucose and citrate can be used that differ in $^{13}$C content on the relative $\delta^{13}$C scale by 15‰, determined with an accuracy of ±0.2‰. Exploiting these differences, it has proved possible to determine the relative utilization of glucose and citrate for lactic acid, diacetyl and acetoin production in growing cultures of *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*.

2. Materials and methods

2.1. Bacterial cultures

*Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* strain B7/2147, obtained from the National Collection of Lactic Acid Bacteria (Institute of Food Research, Norwich, UK), was stored at −80°C in M17 medium [8] with 15% (v/v) glycerol. Routine culture was in sterile (30 min; 121°C; 1 bar) M17 broth (per l: tryptone, 2.5 g; casein peptone, 2.5 g; Papain peptone, 5 g; yeast extract, 2.5 g; sodium glycerophosphate, 19 g; magnesium sulfate, 0.25 g; ascorbic acid, 0.5 g). The bacteria were pre-cultured for 6 h in M17 with glucose (27.8 mM) or glucose+citric acid (27.8 mM+13.9 mM) added by sterile filtration as required. Fermentation was initiated by inoculating 200 ml of sterile M17 with added glucose (27.8 mM) or glucose+citric acid (27.8 mM+13.9 mM) as appropriate in a 200 ml Duran bottle with 0.2 ml of pre-culture. The fermentation conditions were: anaerobic (static, closed), 30°C, pH initially 6.2–6.4 (HCl) and left to evolve freely during 16 h. Cultures were harvested after 16 h, the supernatant recovered by centrifugation (4500 g, 10 min, 4°C) and kept at −20°C.

2.2. Analytical procedures

Acetoin and diacetyl were recovered from the fermentation broth using solid-phase microextraction fibers (Supelco) coated with polydimethylsiloxane/divinylbenzene (PDMS/DVB, 65 μm) or carboxene/polydimethylsiloxane (CAR/PDMS, 75 μm). Medium (25 ml) was placed in a 40-ml vial and saturated with salt (NaCl). The headspace above the vigorously-stirred sample was left for 1 h to equilibrate at 30°C. The fiber was inserted into the headspace and retained for 10 min. Then, the fiber was retracted and directly transferred to the injection port of a GC linked via a combustion interface to an IRMS, as described previously [9].

Lactic acid was isolated from 20 ml of culture filtrate by anion exchange chromatography (Dowex 1X8, Cl−), recuperation being effected with 2 M HCl, followed by HPLC (Lichrosorb RP-18, 10×250 mm, Merck) eluted isocratically with 0.01 M HCl (2 ml min$^{-1}$). The $\delta^{13}$C values were determined by EA-IRMS on encapsulated samples [10]. Purity was confirmed by $^1$H NMR.

Metabolite concentrations in the culture medium were
The kinetics of fermentation of Lactococcus lactis subsp. lactis biovar. diacetylactis strain B7/2147 in glucose/citrate co-fermentation are shown in Fig. 2. An initial pH of 6.2–6.4 was used to avoid the pH-dependent lag in citrate utilization found in fermentations initiated at pH 7.4. Lactic acid, diacetyl and acetoin production could all be detected within the first 2 h of fermentation and the accumulation of all three products reached a plateau within 8 to 10 h, after which the concentrations remained stable for at least another 10 h (data not shown). Available glucose and citrate were both consumed during the phase of exponential growth of the fermentation, with $k_d$ (mM h$^{-1}$) = 4.6 and 2.7, respectively, over the 4–8 h period of growth. Both substrates were at least 90% exhausted by 12 h. Fermentations with glucose alone provided a similar profile, except that negligible amounts of diacetyl and acetoin were found (data not shown). On this basis, 16 h fermentations were used to examine the $\delta^{13}$C ($\%$) values of lactic acid, diacetyl and acetoin.

Values from a typical fermentation with mixed substrates are given in Table 1. As can be seen, the co-metabolism of glucose and citrate led to $\delta^{13}$C ($\%$) values in lactic acid, diacetyl and acetoin intermediate to those of the two available substrates, unequivocally demonstrating that citrate and glucose are being incorporated into both types of product. Other fermentations under slightly different conditions and using alternative methods to extract the diacetyl and acetoin similarly gave intermediate values (data not shown). From fermentations conducted with glucose alone, the mean impoverishment of lactic acid was found to be 0.3 $\%$, indicating there was minimal fractionation between glucose and pyruvate. The variation between cultures grown on C-4 derived glucose ($\delta^{13}$C = −10.0) and C-3 derived glucose ($\delta^{13}$C = −23.6) was probably due to the non-statistical distributions of $^{13}$C in the glucose derived from the C-3 or C-4 sources [16]. Acetoin and diacetyl showed $\delta^{13}$C values close to one another, as might be expected considering their metabolic

Table 1

The measured $\delta^{13}$C ($\%$) values obtained for lactic acid, diacetyl and acetoin from fermentations of Lactococcus lactis subsp. lactis biovar. diacetylactis strain B7/2147 with glucose alone or glucose/citrate co-metabolism

<table>
<thead>
<tr>
<th>Conditions of culture</th>
<th>Lactic acid ($\delta^{13}$C $%$)</th>
<th>Diacetyl ($\delta^{13}$C $%$)</th>
<th>Acetoin ($\delta^{13}$C $%$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose only (5 g/l)</td>
<td>$\delta^{13}$C $%$</td>
<td>$\delta^{13}$C $%$</td>
<td>$\delta^{13}$C $%$</td>
</tr>
<tr>
<td>$-10.0$</td>
<td>$-11.7$ (3)</td>
<td>$-15.8$</td>
<td>$-14.9$</td>
</tr>
<tr>
<td>$-23.6$</td>
<td>$-22.6$ (3)</td>
<td>$-19.2$</td>
<td>$-18.5$</td>
</tr>
<tr>
<td>Glucose (27.8 mM)+Citrate (13.9 mM)</td>
<td>$\delta^{13}$C $%$</td>
<td>$\delta^{13}$C $%$</td>
<td>$\delta^{13}$C $%$</td>
</tr>
<tr>
<td>$-10.0$</td>
<td>$-11.4$</td>
<td>$-11.2$</td>
<td>$-15.8$</td>
</tr>
<tr>
<td>$-24.7$</td>
<td>$-13.3$</td>
<td>$-19.2$</td>
<td>$-18.5$</td>
</tr>
<tr>
<td>$-23.6$</td>
<td>$-11.4$</td>
<td>$-20.7$</td>
<td>$-24.5$</td>
</tr>
<tr>
<td>$-24.7$</td>
<td>$-23.3$</td>
<td>$-29.1$</td>
<td>$-27.4$</td>
</tr>
</tbody>
</table>

Values are means for duplicate fermentations unless indicated otherwise (N), each fermentation being analyzed in triplicate. Intra-fermentation variation was not greater than ±0.4 $\%$ and the range between fermentations not greater than ±0.4 $\%$. Values for diacetyl and acetoin are corrected for the effects of the fibre (see text), +1.1 $\%$ and +0.6 $\%$, respectively.
proximity, with a consistently slightly higher impoverishment in diacetyl.

In order to determine the relative quantitative contributions of the substrates to the products, two correction factors need to be applied, (1) that due to instrumental variation and (2) that due to metabolic impoverishment during metabolism.

1. The SPME technique used to recover diacetyl and acetoin from the fermentation broth introduces a small consistent negative shift in the $\delta^{13}C$ values relative to those determined by EA-IRMS on encapsulated samples [9]. This shift is independent of the concentration present, allowing direct correction factors to be applied for diacetyl and acetoin. For the data in Table 1, these are $+1.1\%e$ and $+0.6\%e$, respectively. These correction factors are measured by passing standards with the experimental samples and correcting against the values obtained for instrument drift and the SPME technique.

2. From fermentations conducted using glucose+ citric acid of similar $\delta^{13}C$ (%e) values (either at $-10.0/-11.4\%e$ or $-23.6/-24.7\%e$) it can be calculated that, relative to the substrates supplied ($\Delta\delta^{13}C$ %e), the diacetyl and acetoin are on average impoverished in $^{13}C$ by 5.0 and 3.7% respectively. In contrast, the lactic acid is slightly enriched in $^{13}C$ by 0.2 %e, a level close to that found with glucose alone. These values represent the intrinsic fractionation due to the isotopic effects between the substrates and the end products. As the lactate value is apparently close to that of the pyruvate present (see above), it can be deduced that the impoverishments measured for diacetyl and acetoin are due to metabolism beyond pyruvate. These correction values are applied to the values given in Table 1 for diacetyl and acetoin from co-fermentations of glucose/citric acid with different $\delta^{13}C$ values to compensate for the intrinsic fractionation between the primary carbon sources and the end products.

Hence, the relative incorporations into the three products can be calculated:

$$X(\%) = 100 \times (\delta^{13}CM - \delta^{13}C_C) / (\delta^{13}CG - \delta^{13}C_C)$$

where $X$=proportion of carbon derived from glucose, $\delta^{13}CM =$ measured value, $\delta^{13}C_G =$ reference value for glucose, $\delta^{13}C_C =$ reference value for citrate

Overall, it is apparent (Table 2) that the level of incorporation of glucose into diacetyl and acetoin in the experimental conditions used is of the same order as the incorporation into lactic acid. Thus, there is no evidence that during fermentation in standard M17 broth with an initial pH of 6.2-6.4 citrate is the preferred source of carbon for diacetyl and acetoin biosynthesis. The proportions of carbon incorporation have previously [6] been estimated from the molar ratios of $^{13}C$ incorporation (determined by $^{13}C$/ $^1H$ heteronuclear coupling) in growing cells (initial pH 6.4) provided with citrate (17.5 mM) and glucose (8.8 mM). By this indirect means, it can be estimated that, in their fermentation conditions, the incorporations from glucose into both lactic acid and acetoin were about half those found in the present study. As, however, the concentration ratio in [6] is 2:1 in favor of citrate while in the present study it is 2:1 in favor of glucose, it is perhaps not surprising that glucose was more strongly incorporated in the present study than in [6]. It is clear that the degree of incorporation of the two carbon sources into the aroma compounds might vary markedly with the fermentation conditions used.

4. Conclusions

Ever since the demonstration in 1939 [17] that lactic acid bacteria were capable of metabolizing citrate, the role of this carbon source in the generation of diacetyl has been an unresolved issue. For the first time, it is unequivocally demonstrated that the diacetyl accumulated during the co-fermentation of glucose+citric acid by actively growing Lactococcus lactis subsp. lactis biovar. diacetylactis is substantially derived from the carbohydrate source. Furthermore, the relative utilization of glucose and citrate for lactic acid accumulation and for diacetyl and acetoin accumulation are approximately equivalent. Glucose and citrate are clearly used simultaneously, as indicated by their kinetics of consumption. Furthermore, all the products are found to start to accumulate early in the culture cycle and to continue to increase in concentration until near the end of the growth period.

Table 2

<table>
<thead>
<tr>
<th>Contribution of glucose to:</th>
<th>Lactic acid (%)</th>
<th>Acetoin (%)</th>
<th>Diacetyl (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Present study</td>
<td>77 ($\pm$ 5)</td>
<td>67 ($\pm$ 6)</td>
<td>69 ($\pm$ 8)</td>
</tr>
<tr>
<td>Ramos et al. [6]</td>
<td>36</td>
<td>28</td>
<td>none</td>
</tr>
</tbody>
</table>

Values are calculated from the fermentations with glucose+citric acid differing in $\delta^{13}C$ values (Table 1). Mean corrections for intrinsic metabolic fractionation for all three products are made from the fermentations with glucose+citric acid of equivalent $\delta^{13}C$ values and assuming a $\delta^{13}C$ value intermediate to those of the glucose and citric acid supplied. The maximal uncertainties in this value can be calculated by assuming that all the product is derived from one or the other carbon source. These are given in parentheses.
Thus, when the carbon flux is summed over the entire period of culture, a simple model emerges in which carbon from both glucose and citrate is continuously fed into the pool of pyruvate and in which this pool is simultaneously and continuously used to supply carbon to both lactic acid and diacetyl/acetoin pathway. Thus, a model in which citrate is the primary source of carbon for diacetyl/acetoin can be rejected. This conclusion is independent of parameters such as the relative rates of consumption of glucose and citric acid and of the relative kinetics of lactate and diacetyl/acetoin accumulation. The model is, however, only valid for a complete fermentation of the available substrates. What cannot be deduced from the present data is whether the partitioning of carbon is constant throughout growth or whether there is a preferential utilization of substrates at earlier stages of culture. As the substrates are initially available at a 2:1 ratio and glucose can provide 2 mol of pyruvate per mol, whereas citrate can only provide one mol per mol, there is a theoretical ‘pyruvate availability’ ratio of 4:1 in favor of glucose. The isotopic analysis at exhaustion (Table 2) of the substrates suggests that nearly 80% of the lactate is derived from glucose, compatible with a completely equivalent utilization of the two sources. For diacetyl/acetoin, only 68% of the carbon is derived from glucose. These differences could be due to different kinetics of production of the compounds or to a slightly unequal utilization of citrate-derived pyruvate at some stage in the growth cycle. To clarify these points, kinetic studies and an examination of environmental and genetic influences on this metabolism are required.

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