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## Energetic analysis of cultures of *Lactobacillus delbrueckii* subsp. *bulgaricus*: identification of the type of control between catabolism and anabolism

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**Abstract** – Growth and energetic parameters of *Lactobacillus delbrueckii* subsp. *bulgaricus* were measured during cultures in synthetic medium, at pH regulated at 6.4 and at free pH. These data enabled the actual biomass yield relative to ATP and the maintenance coefficient to be calculated, and the type of control between catabolism and anabolism to be identified. The cultures of *L. delbrueckii* subsp. *bulgaricus* at pH 6.4 or at a non-regulated pH were characterized by a concomitant and early decrease in both catabolism and anabolism. At a regulated pH, the growth decrease seems to be due to the accumulation of toxic compounds, acting on anabolic reactions. This inhibition led to a decrease in the catabolic flux, albeit to a lower extent than the anabolic flux. In this condition, the culture was characterized by an excess of energy. On the contrary, at free pH, the lactic acid production led to a pH decrease responsible for the slowing-down of the catabolic flux. At the same time, maintaining the internal pH in the acidified medium was more energy-consuming. The consequence of both the decrease in the glycolytic flux and the increase in the energy consumption led to an energy limitation of growth, as shown by the lower value of the maintenance coefficient.

**Lactic acid bacteria / *Lactobacillus delbrueckii* subsp. *bulgaricus* / energetic analysis / catabolism / anabolism / maintenance coefficient**

**Résumé** – Les apports de l'énergétique microbienne à l'analyse des cultures de *Lactobacillus delbrueckii* subsp. *bulgaricus* : identification du type de contrôle entre catabolisme et anabolisme. La croissance et les paramètres énergétiques de *Lactobacillus delbrueckii* subsp. *bulgaricus* sont quantifiés en cinétique de culture en milieu synthétique, à pH régulé à 6,4 et à pH libre. Ces données permettent d'estimer le rendement ponctuel de biomasse par rapport à l'ATP et le coefficient de maintenance, paramètres dont l'analyse permet d'identifier le type de contrôle reliant le catabolisme à l'anabolisme. Lors des cultures de *L. delbrueckii* subsp. *bulgaricus* à pH régulé à 6,4 comme à pH libre, un ralentissement concomitant du catabolisme et de l'anabolisme est observé très précocement en cours de culture. À pH régulé, le ralentissement de croissance semble dû à l'accumulation de produits toxiques, qui affecte en premier lieu l'anabolisme, l'inhibition de l'anabolisme provoquant un rétro-contrôle du flux catabolique, mais dans des proportions moindres que l'anabolisme. La culture est alors en situation d'excédent énergétique. Inversement, à pH libre, la production d'acide lactique provoque une diminution du pH responsable d'un ralentissement du catabolisme. En même temps, l'acidification engendre un coût énergétique supplémentaire pour le

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maintien du pH interne. L'action combinée du ralentissement du flux glycolytique de production d'énergie et de l'augmentation du coût énergétique, provoque finalement une limitation énergétique de la croissance, visualisée en particulier par une faible valeur du coefficient de maintenance.

**Bactérie lactique / *Lactobacillus delbrueckii* subsp. *bulgaricus* / analyse énergétique / catabolisme / anabolisme / coefficient de maintenance**

## 1. INTRODUCTION

Among the lactobacilli used extensively in the food industry, *Lactobacillus delbrueckii* subsp. *bulgaricus* is mainly used as starter culture for cheeses as well as for large-scale fermentation of milk into yogurt, in combination with *Streptococcus thermophilus*. Lactic acid bacteria (LAB) are fastidious micro-organisms subjected to inhibitory effects of various produced compounds, i.e., lactic acid, H<sub>2</sub>O<sub>2</sub>, bacteriocin, and to nutritional limitations, depending on the culture medium used. The growth kinetic profiles of LAB presented in the literature are currently characterized by a decrease in the growth rate during the cultivation time. This is particularly true for *L. delbrueckii* subsp. *bulgaricus* cultivated in synthetic media, for which in addition, growth sometimes stops early on the fermentation, before sugar exhaustion [8, 9, 17, 22]. After an exhaustive analysis of all the possible hypotheses to explain this phenotype, Mercade et al. [17] proposed that this growth arrest was due to the production of one (or more) toxic compound which was inhibitory against anabolic reactions. These results illustrate that lactic acid is far from being either the only or the major toxic compound produced by lactic acid bacteria. Previous results led to similar conclusions, which showed that supernatant fluids of cultures of *L. delbrueckii* contained growth-inhibiting factors other than lactic acid [27], or that the inhibitory effect of lactic acid on growth did not reflect the phenotype observed during cultivation of *L. delbrueckii* subsp. *bulgaricus* [1], *L. lactis* subsp. *lactis* [14], or *L. lactis* subsp. *cremoris* [2].

In most cases, the catabolic and anabolic fluxes decrease together when the bacterium is faced with unfavorable growth conditions, albeit to different extents. This is observed when comparing the phenotype of *L. lactis* in culture media of different composition where anabolism is clearly the limiting factor [20], or with different carbon sources leading to various catabolic fluxes [6]. One notable exception is *Streptococcus bovis* which maintains a high catabolic flux whatever the growth rate in media of variable composition, and in consequence evacuates the excess of energy as heat [24]. With regard to the acidification of the medium, a partial uncoupling between catabolism and anabolism is observed when the pH is lowered, in batch cultures at different pH [16], or in a batch at non-regulated pH where the strain is subjected to auto-acidification [5].

In this study, an industrial *L. delbrueckii* subsp. *bulgaricus* strain was grown in a synthetic medium with two pH conditions, regulated at 6.4 or not, and the kinetics of growth and metabolic activity and the energetic parameters were analyzed. From these data, the parameters related to ATP production and consumption, and the maintenance coefficients, were calculated to identify the type of control between catabolism and anabolism.

## 2. MATERIALS AND METHODS

### 2.1. Organism and culture media

*L. delbrueckii* subsp. *bulgaricus* strain Ext2.215 was used throughout this study. The strain was stored at -80 °C in MRS medium supplemented with glycerol (20%).

Growth was studied in a synthetic medium [17], modified from the MPL medium described by Chervaux et al. [4]. This medium, mMPL medium, was prepared from freshly prepared concentrated solutions, and sterilized by filtration through cellulose nitrate membranes (0.22  $\mu\text{m}$  pore size; Sartorius, Gottingen, Germany) directly into the sterilized (20 min at 121 °C) culture vessel.

## 2.2. Growth conditions

Cultures were grown under anaerobic conditions in butyl-rubber-stoppered tubes or in 2-L fermentor (Setric Génie Industriel, Toulouse, France) and at a temperature of 42 °C. The pH was not regulated, or maintained at 6.4 by automatic addition of KOH (10 N), depending on the cultures, as stated in the text. The bacteria were grown under a controlled gas environment by flushing both the vessel and the medium with nitrogen. The medium in the fermentor was aseptically gassed (30 min) immediately before inoculation and maintained under a  $\text{N}_2$  atmosphere at a positive pressure of 20 mbar. Inoculation was at 2% with exponential-phase cells from precultures grown on the same medium.

## 2.3. Analytical methods

Bacterial growth was monitored spectrophotometrically at 580 nm and calibrated against cell dry weight measurements. A change of 1 U of density was shown to be equivalent to 0.27 g of dried matter per liter. Lactose, galactose, glucose and lactate were determined by high-pressure liquid chromatography as previously described [6].

## 2.4. Measurement of $\Delta\text{pH}$ and $\Delta\psi$ values, and of ATPase activity

The transmembrane pH and electrical gradients, e.g.,  $\Delta\text{pH}$  and  $\Delta\psi$  were measured by determining the internal to external gradient of  $^{14}\text{C}$ -benzoic acid and

$^3\text{H}$ -tetraphenylphosphonium bromide ( $\text{TPP}^+$ ), respectively, after centrifugation of the cells through silicon oil, as previously described [13]. The ATPase activity was determined as previously described [5].

## 3. RESULTS

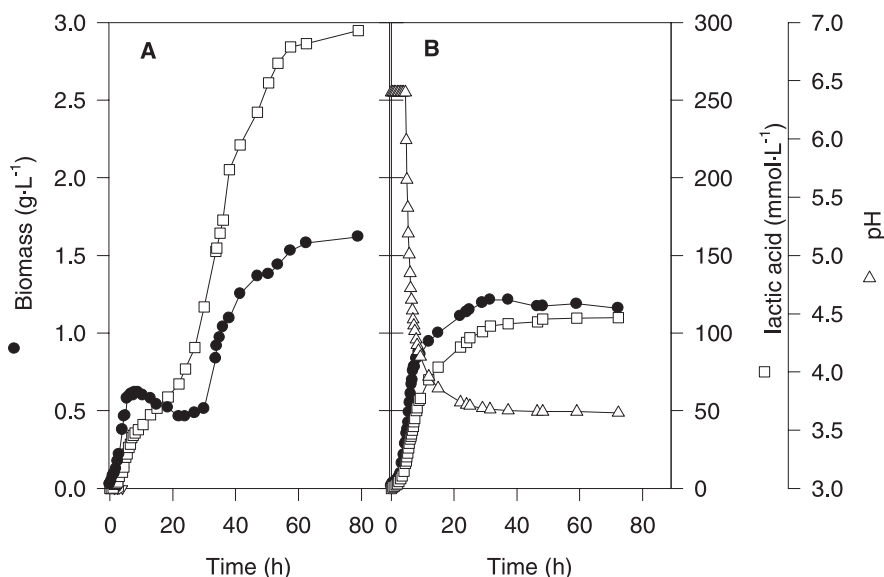
### 3.1. Kinetics of *L. delbrueckii* subsp. *bulgaricus* EXT2.215 in mMPL medium

*L. delbrueckii* subsp. *bulgaricus* Ext2.215 was cultivated in the mMPL medium at pH regulated at 6.4 or at free pH. At regulated pH, no more catabolic sugar remained in the medium after 80 h of cultivation. Lactic acid was the only end-product detected in the medium (Fig. 1A). Growth proceeded in two distinct phases separated by a stationary phase of about 20 h. Catabolic activity continued during this stationary phase, albeit at a slower rate, as shown by the slower increase in lactate accumulation. This transient stop of growth was due to the production during the first phase of growth of toxic compounds, which disappeared later in the culture, as proposed by Mercade et al. [17].

At non-regulated pH, the growth curve exhibited a more classical profile, characterized by a slowing-down of the growth rate when the pH decreased. The growth stopped when the pH reached the value of 3.7 (Fig. 1B). The lactic acid production profile was similar to the growth profile. The residual lactose and glucose concentrations at the time of the growth arrest were 58 and 15  $\text{mmol}\cdot\text{L}^{-1}$ , respectively.

### 3.2. Energetics of *L. delbrueckii* subsp. *bulgaricus* EXT2.215 in mMPL medium

Since in lactic acid bacteria the vast majority of the carbon source is catabolized through the glycolytic pathway, and no other significant energy sources are known, the specific production rate of ATP (qATP)



**Figure 1.** Fermentation time course for *Lactobacillus delbrueckii* subsp. *bulgaricus* Ext2.215 growing on mMPL medium. A: at pH regulated at 6.4; and B: at non-regulated pH.

**Table I.** Growth rate ( $\mu$ ), specific production rate of ATP ( $q_{\text{ATP}}$ ), pH and electrical ( $\Delta\psi$ ) gradients, proton-motive force (PMF), internal pH ( $\text{pH}_{\text{in}}$ ), and ATPase activity, measured at different cultivation times during a culture of *Lactobacillus delbrueckii* subsp. *bulgaricus* Ext2.215 growing on mMPL at pH regulated at 6.4.

Time (h)	4	5.5	7.5	30	34	53.5
$\mu$ ( $\text{h}^{-1}$ )	0.53	0.16	0	0.02	0.15	0.01
$q_{\text{ATP}}$ ( $\text{mmol}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$ )	15	10	5	14	11	1.3
$\Delta\text{pH}$	0.1	0.1	0.1	0.6	0.6	0.6
$\Delta\psi$ (mV)	-31	-29	-29	-50	-44	-45
PMF (mV)	-39	-34	-29	-85	-74	-77
$\text{pH}_{\text{in}}$	6.5	6.5	6.5	7	7	7
ATPase (U)	0.23 $\pm 0.04$	0.17 $\pm 0.03$	0.15 $\pm 0.02$	0.28 $\pm 0.07$	0.14 $\pm 0.01$	0.17 $\pm 0.02$

was considered to be equal to the specific production rate of lactate. In a culture of *L. delbrueckii* subsp. *bulgaricus* Ext2.215 at a pH regulated at 6.4, the specific production rate of ATP ( $q_{\text{ATP}}$ ) decreased gradually during the first growth phase, increased to its maximal level at the onset of the second growth phase, and then decreased again

during the second growth phase (Tab. I). The pH gradient between the intracellular content and the culture broth was very low during the first growth phase, and it increased to 0.6 units later in the culture, despite the constant pH of the medium. A concomitant increase was observed for the electrical gradient, and in consequence,

**Table II.** Growth rate ( $\mu$ ), pH of the broth ( $\text{pH}_{\text{out}}$ ), pH and electrical ( $\Delta\psi$ ) gradients, proton-motive force (PMF), internal pH ( $\text{pH}_{\text{in}}$ ), ATPase activity, and activity of ATPase corrected by the effect of internal pH on the activity (Corrected ATPase), measured at different cultivation times during a culture of *Lactobacillus delbrueckii* subsp. *bulgaricus* Ext2.215 growing on mMPL at non-regulated pH.

Time (h)	4.5	6	8	24	48	72
$\mu$ ( $\text{h}^{-1}$ )	0.6	0.38	0.07	0.01	0	0
$q_{\text{ATP}}$ ( $\text{mmol}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$ )	31.4	19	12.5	1.4	0.2	0
$\text{pH}_{\text{out}}$	6.4	4.85	4.23	3.72	3.66	3.65
$\Delta\text{pH}$	0.1	0.48	0.65	0.99	1.06	1.01
$\Delta\psi$ (mV)	-30	-26	-26.3	-31.8	-48.9	-45.5
PMF (mV)	-35.9	-54.3	-64.6	-90.2	-111.4	-105.1
$\text{pH}_{\text{in}}$	6.5	5.33	4.9	4.71	4.72	4.66
ATPase (U)	0.16	0.22	0.30	0.20	0.02	0.02
	$\pm 0.02$	$\pm 0.02$	$\pm 0.03$	$\pm 0.03$	$\pm 0.01$	$\pm 0.01$
Corrected ATPase (U)	0.12	0.08	0.08	0.05	0.005	0.004

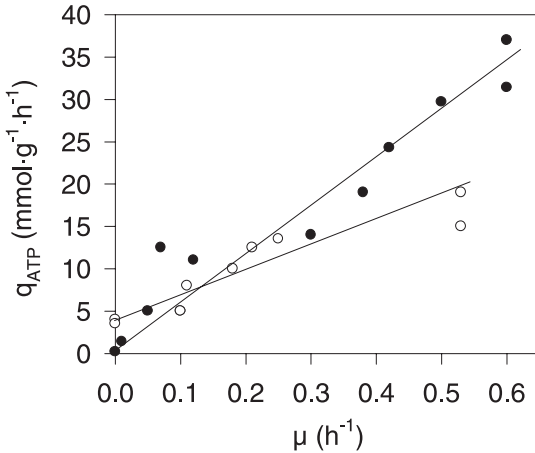
the global proton-motive force (PMF) increased from  $-35$  to  $-80$  mV between the different phases (Tab. I). The ATPase activity did not vary significantly during the culture.

In a culture at free pH,  $q_{\text{ATP}}$  decreased concomitantly to the growth rate, due to the gradual slowing-down of the lactate production (Tab. II). The initial pH gradient was logically identical to that previously observed, at a very low value. However, while the pH of the culture broth decreased, the  $\Delta\text{pH}$  value increased throughout the growth phase to reach a maximal value of about 1 unit during the stationary phase (Tab. II). At the arrest of growth, the internal pH was 4.7. The  $\Delta\psi$  value remained constant during the growth phase, while the  $\Delta\text{pH}$  increased, and then increased a little during the stationary phase. Due to the  $\Delta\text{pH}$  increase, the PMF increased throughout the culture, from  $-35$  to about  $-110$  mV in the stationary phase. The ATPase activity during the growth phase was similar to that previously determined at regulated pH, but a strong decrease was observed in the stationary phase.

#### 4. DISCUSSION

The cultures of *L. delbrueckii* subsp. *bulgaricus* presented above are characterized by an early and gradual decrease in both the growth rate and the catabolic flux during the growth phase. It appears clearly from the literature that such a phenotype can be imposed by different causes, and thus, it is often difficult to identify the metabolic events responsible for the observed behavior. A complete analysis of the cultures, including all the growth, metabolic and energetic parameters available, and the putative relationships between them, is necessary to explain the behavior of the bacterium.

At the beginning of growth of *L. delbrueckii* subsp. *bulgaricus* in mMPL medium, a very short exponential growth is observed, the maximal growth rate observed in this condition being characteristic of the nutritional and environmental conditions maintained in the culture. Since in a more complex medium the maximal growth rate is higher than in mMPL medium ( $\mu_{\text{max}} = 0.94 \text{ h}^{-1}$  in MRS medium,



**Figure 2.** Relationship between the specific rate of ATP production ( $q_{\text{ATP}}$ ) and the growth rate ( $\mu$ ) in cultures of *Lactobacillus delbrueckii* subsp. *bulgaricus* Ext2.215 growing on mMPL medium. ○: at pH regulated at 6.4; and ●: at non-regulated pH.

data not shown), one can assume that the growth rate in mMPL is limited by anabolism, as demonstrated for the growth of *Lactococcus lactis* in the same kind of synthetic medium [20]. After this short exponential growth phase, the decrease in the growth rate is considered to be due to an inhibition of anabolism linked to the accumulation of toxic compounds [17]. This growth decrease is accompanied by a slowing-down of the energy-producing catabolic flux converting the sugar into lactic acid, leading to a linear relationship linking the specific rate of ATP production ( $q_{\text{ATP}}$ ) and the growth rate (Fig. 2).

Analyzing the comparative evolution of the energy-producing catabolic flux and the growth rate, i.e., the anabolic flux, in different culture conditions, has been known since 1965 as a good way to identify the type of control between catabolism and anabolism [23]. The  $q_{\text{ATP}}$  and the growth rate are related by the following equation:

$q_{\text{ATP}} = \mu / Y_{\text{ATP}}^{\text{max}} + m_{\text{ATP}}$ , where  $Y_{\text{ATP}}^{\text{max}}$  is the maximal biomass yield relative to ATP, and  $m_{\text{ATP}}$  is the part of ATP used for maintenance, i.e., non-growth functions. This first simple equation has been subsequently improved [21, 25] to take into account the fact that the maintenance could vary with the growth rate:

$q_{\text{ATP}} = \mu / Y_{\text{ATP}}^{\text{max}} + m_{\text{ATP}} + m_g \times \mu$ , where  $m_g \times \mu$  is the part of the maintenance energy which is proportional to the growth rate. This relation indicates that the global maintenance should decrease when the growth rate diminishes.

The relation linking  $q_{\text{ATP}}$  and  $\mu$  established for the growth of *L. delbrueckii* subsp. *bulgaricus* Ext2.215 at pH regulated at 6.4 (Fig. 2) enables the actual ATP yield and the apparent maintenance coefficient to be calculated. The actual ATP yield ( $Y_{\text{ATP}} = \mu / q_{\text{ATP}}$ ) represents the biomass effectively synthesized per mole of ATP, without taking into account the part of ATP used for maintenance functions. From Figure 2, the calculation of the actual  $Y_{\text{ATP}}$  for different values of growth rate shows that  $Y_{\text{ATP}}$  decreases when  $\mu$  diminishes:  $Y_{\text{ATP}} = 25.1, 23.1, 20.2,$  and  $14.5 \text{ g}\cdot\text{mol}^{-1}$  at  $\mu = 0.4, 0.3, 0.2,$  and  $0.1 \text{ h}^{-1}$ , respectively. The fact that the actual  $Y_{\text{ATP}}$  diminishes during the culture when  $\mu$  decreases indicates that the decrease in the energy-producing flux ( $q_{\text{ATP}}$ ) is less pronounced than the decrease in the anabolic flux ( $\mu$ ), and thus that the global maintenance coefficient increases. For the values of growth rate presented above, the maintenance coefficient ( $m_{\text{ATP}}$ ) increases from 2.6 to 3.0, 3.2 and 3.6  $\text{mmol}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$ ,

respectively. This finding seems to be logical if we consider that the growth is progressively inhibited during the culture. This behavior cannot be accounted for by the previous relationship linking  $q_{\text{ATP}}$  and  $\mu$ . In consequence, in the particular case where the decrease in the growth rate is due to an inhibition of anabolic functions, the relationship between  $q_{\text{ATP}}$  and  $\mu$  should be completed by a maintenance coefficient inversely proportional to the growth rate. The following relationship is proposed to take into account this phenomenon:

$q_{\text{ATP}} = \mu / Y_{\text{ATP}}^{\text{max}} + m_{\text{ATP}} + m_{\text{g}} \times \mu + m_{\text{i}}(\mu_{\text{max}} - \mu)$ , where  $m_{\text{ATP}}$  is the basal maintenance,  $m_{\text{g}} \times \mu$  is the part of the maintenance proportional to the growth rate, and  $m_{\text{i}}(\mu_{\text{max}} - \mu)$  is a maintenance coefficient illustrating an energetic uncoupling resulting from a growth inhibition. The coefficient  $m_{\text{i}}(\mu_{\text{max}} - \mu)$  can illustrate an inhibition of growth for energetic reasons, for example, when the growth is inhibited by a weak organic acid, and is in this case a true energetic uncoupling, or can illustrate a coarse adjustment between the flux of energy production and consumption, and thus an energy waste probably as futile cycles. The main question here is how to differentiate between these two hypotheses.

The estimation of the apparent maintenance coefficient, e.g., the value of  $q_{\text{ATP}}$  when  $\mu$  is zero, gives the value of  $4 \text{ mmol}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$ , a value which is clearly overestimated since during the second growth phase, growth is observed while the  $q_{\text{ATP}}$  is as low as  $1.3 \text{ mmol}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$  (Tab. I). This kind of estimation of  $m_{\text{ATP}}$  can be considered to be correct only if the culture is energy-limited [26], indicating in this case that the use of energy is optimized for growth functions. From these data, one can conclude that the culture of *L. delbrueckii* subsp. *bulgaricus* Ext2.215 at regulated pH is inhibited at the level of anabolism, but not energy-limited, indicating that the toxic effect of the produced inhibitory compounds is not an energetic uncoupling

effect like the action of organic acids, and that the catabolic flux decreases to fit the anabolic capabilities, but with a poor adjustment, leading to an energy excess. This conclusion can be reinforced by the observation of the PMF during the culture. While the value of the PMF is very low at the beginning of growth comparing with other anaerobic bacteria [3, 15], probably indicating that the optimal internal pH for growth is about 6.5, this value increases later in the culture, despite the fact that the external pH is maintained constant, that lactic acid is produced, and that the growth rate has diminished. For acidogenic bacteria confronted with the accumulation of organic acids, the  $\Delta\text{pH}$  and the PMF tend to decrease during the culture [12]. In the culture of *L. delbrueckii* subsp. *bulgaricus* Ext2.215, the increase in the PMF could be explained by the excess of energy postulated for the second part of the culture, and it shows that lactic acid does not have a determining effect on the culture behavior.

In a culture at non-regulated pH, the initial  $\Delta\text{pH}$  was as low as during the previous culture at regulated pH, confirming that this value was a characteristic of this bacterium. Both the external and the internal pH decreased concomitantly during the culture (Tab. II), as previously known [11], but to different extents, leading to an increase in the  $\Delta\text{pH}$ . This increase enables the internal pH to be maintained at values compatible with growth and survival [7, 18]. The growth rate decreased gradually during the culture, as did the specific rate of energy production ( $q_{\text{ATP}}$ ), and finally, the growth ceased when the external pH was about 3.7, and the internal pH 4.7, but the true cause of the halt of growth remained to be elucidated. Different hypotheses could be postulated to explain the growth arrest in this culture: (i) an anabolic inhibition by toxic compounds as for the culture at non-regulated pH; (ii) a classical toxic effect of the accumulated lactic acid at low pH, in the form of a protonophore action, i.e., a decrease in the pH gradient, leading to an energetic uncoupling; or (iii) a decrease in



the catabolic rate due to the fall of internal pH, leading to a true energetic limitation. When looking at the relationship between  $q_{\text{ATP}}$  and  $\mu$  deduced from the culture at free pH, the estimation of  $m_{\text{ATP}}$  gives a value of  $0.4 \text{ mmol}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$  (Fig. 2). While the maintenance value for the culture at regulated pH was clearly overestimated, at free pH the value calculated was representative of an energy-limited culture. Contrarily to the culture at constant pH, the relationship between  $q_{\text{ATP}}$  and  $\mu$  at free pH indicates that the energy-producing catabolic activity is the first function which is inhibited by the pH drop. This assumption was confirmed by the inhibitory effect of the low internal pH on glycolytic enzyme activities (data not shown). The energy production is then progressively decreased, while in parallel, lowering the pH, increasing the inhibitory effect of lactic acid at low pH, and improving the PMF add an additional energy cost to the cell [5, 16]. At the time of the halt of growth, the residual catabolic activity is just enough to gain energy for the maintenance functions.

Lowering the internal pH, and its effect on glycolytic enzymes, seems to be the crucial phenomenon responsible for the fall of growth in *L. delbrueckii* subsp. *bulgaricus* Ext2.215 at free pH. This conclusion poses the question of the role of the ATPase in maintaining the internal pH, since in anaerobic bacteria, the major way to improve the internal pH is by proton extrusion through the activity of this enzyme. The in vitro specific activity of the ATPase of *L. delbrueckii* subsp. *bulgaricus* Ext2.215 is not significantly affected by the pH, as in *Lactobacillus casei* [10, 19], since its activity remained constant throughout the growth phase while the pH decreased considerably. With regard to the ATPase activity, a distinction can be made between the growth and stationary phases, since the specific activity of this enzyme falls dramatically during the stationary phase while the pH does not significantly change compared with the end of growth. As for glycolytic enzymes,

the ATPase activity is subjected to pH values (data not shown), and the in vitro activity must be corrected by this effect to estimate the in vivo activity (Tab. II). While the apparent ATPase activity was constant during the growth phase, its in vivo activity gradually decreased due to the medium acidification. Finally, in addition to the decrease in ATP production due to the inhibition of glycolytic enzymes by the internal pH, the simultaneous diminution for the same reason in the ATPase activity should participate in the global growth inhibition by acidification.

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