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Effects of Aeration on Growth and on Production of Bacteriocins and Other Metabolites in Cultures of Eight Strains of Lactic Acid Bacteria

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Abstract

In general, it is accepted that the production of bacteriocins in lactic acid bacteria cultures implies moderately to highly restrictive conditions regarding the availability of oxygen. However, the situation appears to be more complex, probably owing to the facultative anaerobic character of these microorganisms. By studying the culture of eight strains of lactic acid bacteria carried out in vessels with different loads of medium within an interval that determines linearly the minimum availability of oxygen, the existence of three types of behavior was highlighted: production increases (1) with the availability of oxygen, (2) with the restriction of this availability, and (3) toward both extremes of these conditions, diminishing in intermediate situations. These behaviors affected not only the production of bacteriocins, but also their metabolic character (in the Luedeking and Piret sense), as well as the production of other characteristic metabolites, such as lactic acid, acetic acid, and ethanol.

Index Entries: Aeration; bacteriocin production; lactic acid bacteria; aerobiosis; metabolites.

Introduction

Lactic acid bacteria are facultative anaerobic microorganisms; that is, they are capable of modifying a central part of their metabolic pathways

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according to the availability of oxygen in the medium, which allows their classification into homo- and heterolactics. In the first (typically lactococci), lactic acid is always the only final product of fermentation; in the second, lactic acid appears accompanied, in aerobiosis, by different proportions of several oxidized metabolites (acetic acid, diacetyl, acetoin, ethanol, 2,3-butanediol). Thus, rather than two bacterial types, the homo- or heterolactic character appears to define two metabolic forms, homo- and heterofermentative. In fact, Thomas *(1,2)*, among other investigators, observed the change from one form to the other when working with *Streptococcus lactis* in aerobiosis at low glucose levels.

Bacteriocins are peptides with antibiotic properties, which are of growing interest in the food industry and most characteristic of lactic acid bacteria. It seems reasonable to suppose, then, that the production of bacteriocins may be affected by conditions of aeration, a hypothesis to which the disparity of conditions, which from this point of view are described (though not always explicitly mentioned) in the literature, contributes. In fact, although cultures are often carried out disregarding control or measurement of the oxygen level, the aforementioned production appears to be linked to conditions of both strict anaerobiosis and aerobiosis, which at times must be intense, given the low load and the agitation imposed on the Erlenmeyer flasks used in the experiment. In fact, divergent results can even be found relating to the same strains. For example, whereas Hirsch *(3)*, De Vuyst *(4)*, and Kim et al. *(5)* note that the production of nisin by *Lactococcus lactis* requires anaerobiosis, or that "typical conditions" imply moderate agitation or absence of agitation, Cabo et al. *(6)* find, with the same strain, maximum production under clearly aerobic conditions.

To deal with this problem, we compared the time course of the most characteristic descriptive variables of the cultures of these microorganisms in eight strains of lactic acid bacteria (*Lactobacillus brevis*, *Lactobacillus casei* [two strains], *Lactobacillus helveticus*, *L. lactis*, *Leuconostoc mesenteroides*, *Pediococcus acidilactici*, and *Carnobacterium piscicola*) growing under different aeration conditions. These conditions were obtained by using different loads of medium in similarly shaped containers (50, 80, 120, 160, and 200 mL of MRS medium in 250-mL Erlenmeyer flasks, customary in the search for optimum conditions). Thus, a series of situations was defined with increasing restrictions to mass transfer, which in the case of oxygen may be measured in terms of its saturation percentage in the medium, by means of a galvanometric electrode.

Materials and Methods

Microbiologic Methods

Table 1 provides the strains used, together with their sources. Stock cultures were stored at –50°C in powdered skimmed milk suspension with 25% glycerol *(7)*. Inocula (1% [v/v]) consisted of cellular suspensions from

Table 1
Strains Used

Strain	Reference[a]	Abbreviated key
Lactobacillus brevis	CECT 216	Lb 2.01
Lactobacillus casei spp. *casei*	CECT 4040	Lb 3.03
Lactobacillus casei spp. *casei*	CECT 4043	Lb 3.04
Lactobacillus helveticus	CECT 541	Lb 6.04
Lactococcus lactis spp. *lactis*	CECT 539	Lc 1.04
Leuconostoc mesenteroides spp. *mesenteroides*	CECT 4046	Ln 3.07
Pediococcus acidilactici	NRRL B-5627	Pc 1.02
Carnobacterium piscicola	CECT 4020	Cb 1.01

[a]CECT, Spanish Type Culture Collection (Colección Española de Cultivos Tipo); NRRL, Northern Regional Research Laboratory (Peoria, IL).

12 to 24-h-aged cultures on MRS medium, adjusted to an absorbance (700 nm) of 0.900. Cultures were grown in 250-mL Erlenmeyer flasks with 50, 80, 120, 160, and 200 mL of MRS medium at 30°C and 200 rpm of orbital shaking. All assays were carried out in triplicate.

Analytical Methods

At preestablished times, each culture sample was divided into two aliquots. The first was centrifuged at 3220*g* for 15 min, and the sediment was washed twice and resuspended in distilled water to the adequate dilution for measuring the absorbance at 700 nm. The dry weight could then be estimated from a previous calibration curve for each strain. The corresponding supernatant was used for the determination of reducing sugars *(8)*; proteins *(9)*; and lactic acid, acetic acid, and ethanol by high-performance liquid chromatography analysis (refractive index detector) using an ION-300 column (Interaction Chromatography) with 6 m*M* sulfuric acid as a mobile phase (flow = 0.4 mL/min) at 65°C. The second aliquot was used for the extraction and quantification of bacteriocin, according to methods described in detail in previous works *(7,10)*, using *C. piscicola* CECT 4020 as an indicator. Oxygen pressure was determined as a percentage of the saturation value in the same medium, by galvanometry during each sampling period without stopping agitation. All assays were carried out in triplicate.

Numerical Methods

Fitting procedures and parametric estimations calculated from the experimental results were carried out by minimization of the sum of quadratic differences between observed and model-predicted values using the nonlinear least-squares (quasi-Newton) method provided by the macro Solver of the Microsoft Excel 97 spreadsheet.

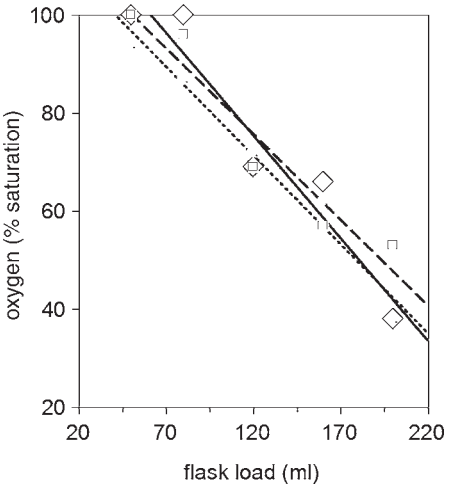

Fig. 1. Relationships between flask load and minimum pO_2 in the three strains (□: Lb 2.01; ○: Pc 1.02; ◇: Lb 6.04) representative of the three types of response to oxygen availability. Dotted lines represent experimental values and solid lines represent fits to simple linear models.

Results and Discussion

Types of Response to Degree of Aeration

As was expected, the loads of the recipients clearly conditioned the time course of the oxygen saturation percentages (pO_2), whose values were minimum approximately at the time when maximum biomass was reached, never dropping below 30%. Figure 1 shows the dependency between the load in the receptacle recipients and the pO_2 minimum for the three strains representative of the three behaviors that are defined subsequently and that adjust acceptably within the period studied to linear models with statistically indistinguishable parameters ($\alpha < 0.05$) in the three cases. Under these conditions, it may be accepted that the loads in the container recipients may be understood with reasonable exactness to be an indicator negatively correlated with the availability of oxygen during the most critical phases of the corresponding cultures.

The responses detected on variation of this availability, although depending on the strains considered, allowed the definition of certain regular patterns of interest. These regular patterns are reflected primarily in the concavity or convexity of the profiles that they represent according to the load in the container recipients, the consumption of nutrients, and the characteristic yield of lactic acid bacteria measured at the time of maximum biomass. Taking into account all of the information shown in Figs. 2–4 (representative of all the groups studied, whose characterization in terms of kinetic parameters is shown in Table 2), it is possible to define three basic types of response to the degree of aerobiosis, which are discussed next.

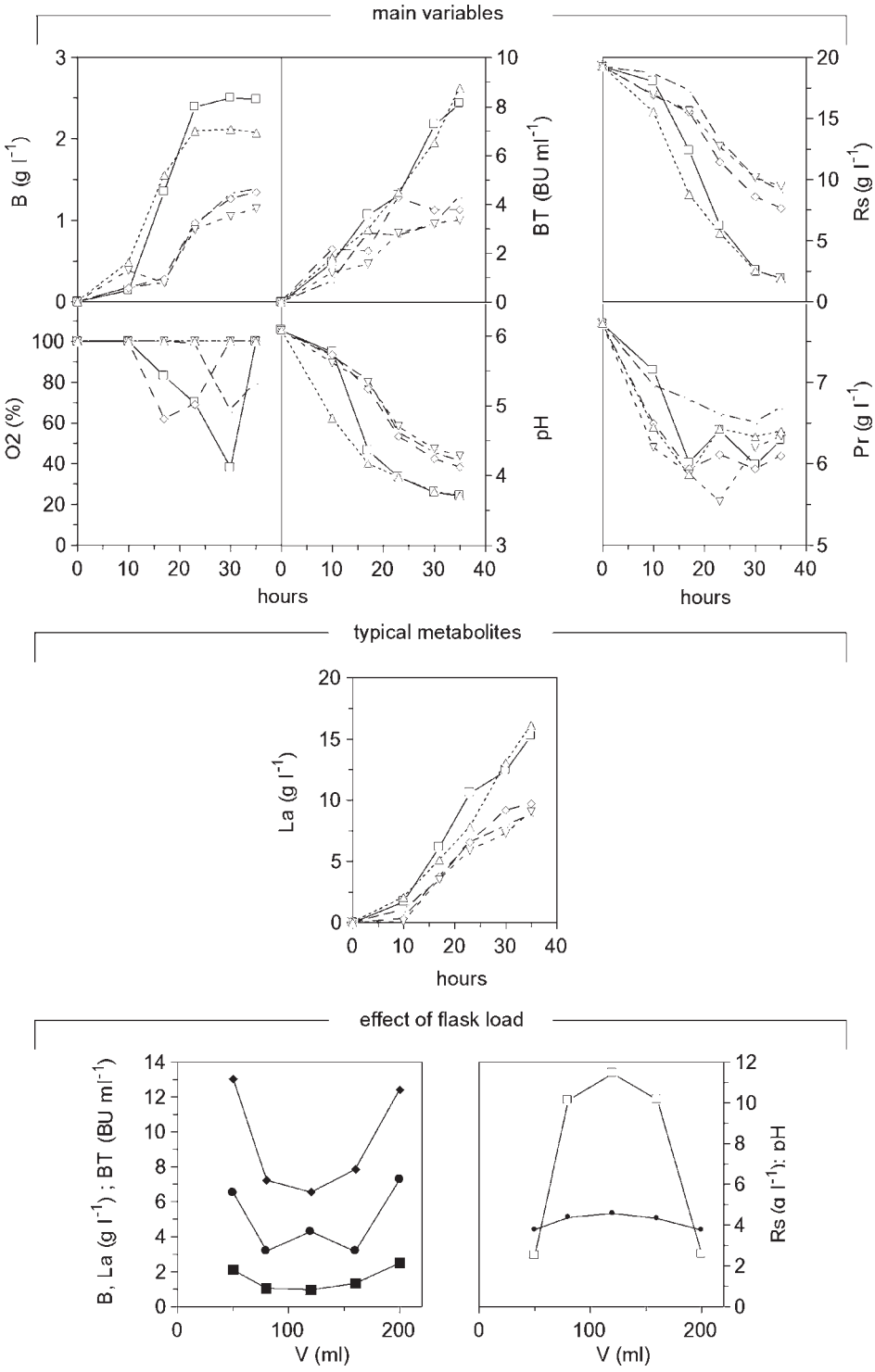

Fig. 2. Top and middle: Time course of *L. brevis* cultures at different flask loads (□: 200, ○: 160, ◇: 120, ▽: 80, and △: 50 mL). B, biomass; BT, bacteriocin; Rs, reducing sugars; Pr, proteins; La, lactic acid. Bottom: Effect of flask load (V) on (•) pH, (■) biomass, (●) bacteriocin, (◆) lactic acid, and (□) consumption of reducing sugars.

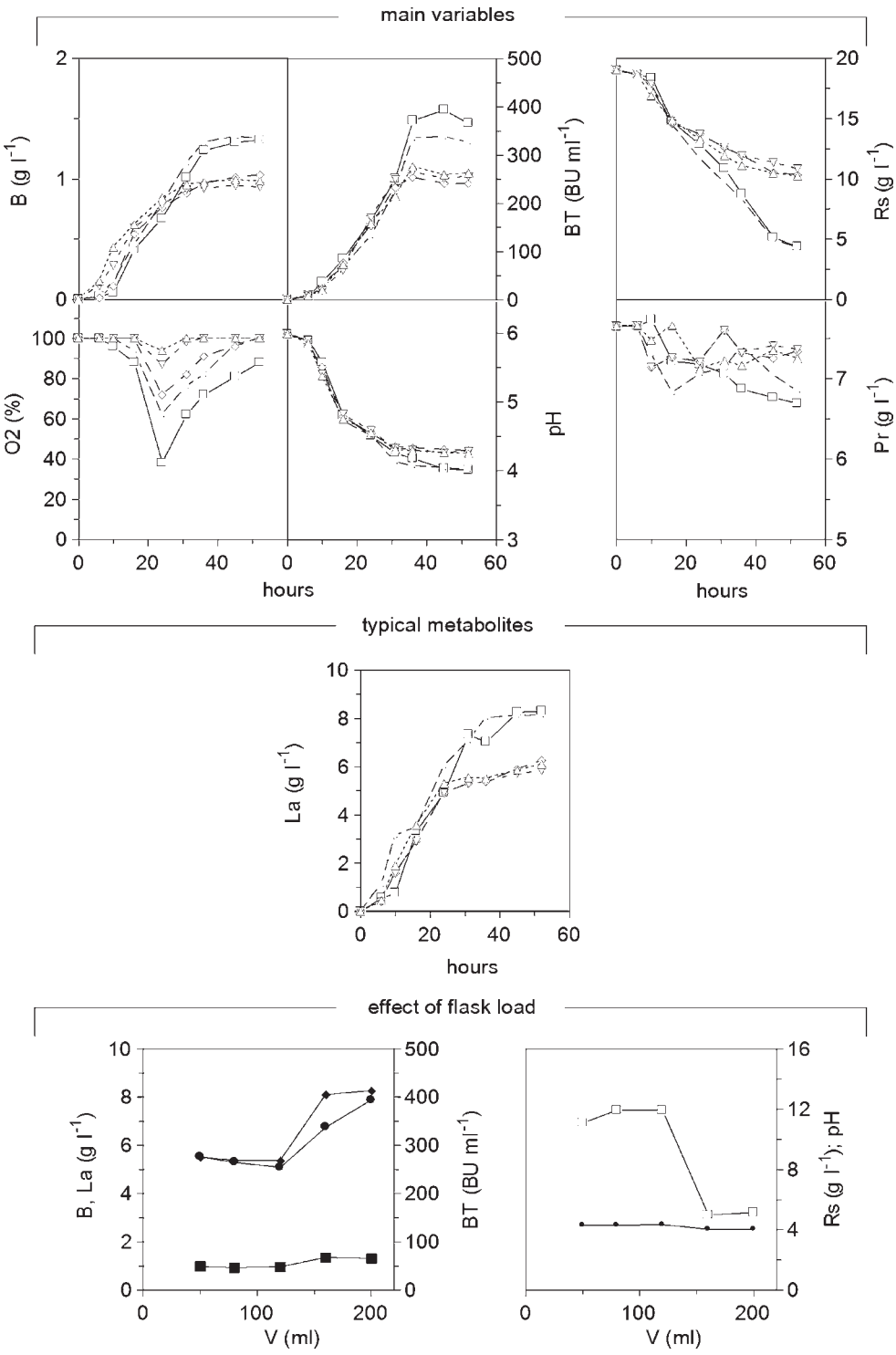

Fig. 3. Top and middle: Time course of *P. acidilactici* cultures at different flask loads (□: 200, ○: 160, ◇: 120, ▽: 80, and △: 50 mL). B, biomass; BT, bacteriocin; Rs, reducing sugars; Pr, proteins; La, lactic acid. Bottom: Effect of flask load (V) on (•) pH, (■) biomass, (●) bacteriocin, (◆) lactic acid, and (□) consumption of reducing sugars.

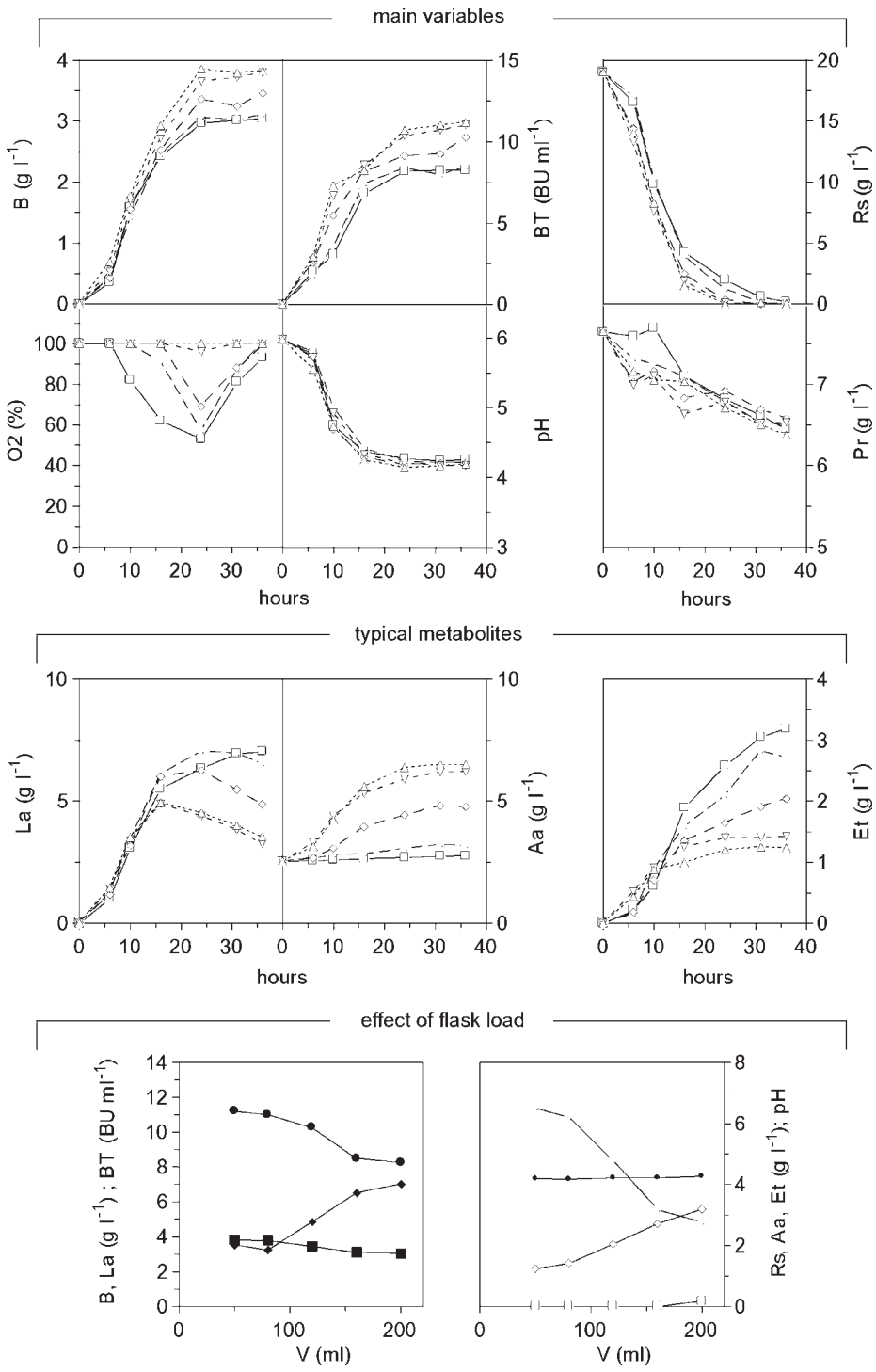

Fig. 4. Top and middle: Time course of *L. helveticus* cultures at different flask loads (□: 200, ○: 160, ◇: 120, ▽: 80, and △: 50 mL). B, biomass; BT, bacteriocin; Rs, reducing sugars; Pr, proteins; La, lactic acid; Aa, acetic acid; Et, ethanol. Bottom: Effect of flask load (V) on (•) pH, (■) biomass, (●) bacteriocin, (◆) lactic acid, (○) acetic acid, (◇) ethanol, and (□) consumption of reducing sugars.

Table 2
Main Parametric Estimations (as Defined in Nomenclature)
of Kinetic Models (Eqs. 5 and 6)
Describing Biomass and Bacteriocin Production
by Lactic Acid Bacteria Growing in Cultures with Different Load Volumes[a]

	K	μ_m	α	β	$Y_{B/G}$	$Y_{BT/B}$	$Y_{BT/G}$	BT_m [a]
Lb 2.01								
50	2.118	0.335	7.594	0.876	0.119	4227	504	8.75
80	1.279	0.163	22.493	—	0.116	2930	340	3.34
120	1.344	0.319	21.656	—	0.116	2821	326	4.29
160	1.392	0.342	18.929	—	0.136	3130	425	4.32
200	2.502	0.451	11.088	0.528	0.143	3278	469	8.13
Lb 3.03								
50	1.785	0.281	34.962	—	0.103	4848	498	8.61
80	1.871	0.278	28.526	—	0.108	4153	450	7.75
120	0.858	0.229	36.724	—	0.089	4529	401	4.14
160	1.183	0.219	29.261	—	0.089	3570	319	4.74
200	2.150	0.236	14.933	0.325	0.123	3128	386	6.70
Lb 3.04								
50	1.722	0.294	31.394	0.648	0.108	7226	782	12.14
80	1.581	0.141	36.037	0.168	0.105	6105	642	7.57
120	1.436	0.277	17.170	1.022	0.103	5219	538	7.15
160	1.765	0.276	30.277	0.605	0.135	6186	834	10.33
200	1.969	0.291	25.004	0.449	0.125	4984	625	9.47
Lb 6.04								
50	3.835	0.280	16.944	—	0.201	2935	589	11.21
80	3.748	0.270	17.449	—	0.199	2904	578	11.00
120	3.355	0.292	16.962	—	0.181	2977	540	10.27
160	3.058	0.325	15.081	0.016	0.163	2737	446	8.48
200	2.978	0.349	12.970	0.125	0.161	2718	438	8.26
Lc 1.04								
50	0.615	0.904	71.633	1.098	0.115	17,969	2064	12.54
80	0.641	0.982	71.352	0.636	0.101	17,103	1731	11.76
120	0.567	1.417	71.314	0.748	0.082	18,737	1543	11.48
160	0.563	1.559	75.983	1.087	0.080	21,679	1744	12.37
200	0.572	1.052	83.654	0.944	0.094	22,105	2076	12.60
Ln 3.07								
50	4.087	0.396	11.913	—	0.210	2250	473	9.41
80	4.071	0.396	9.981	0.041	0.213	2083	443	8.43
120	3.886	0.373	11.743	0.043	0.206	2362	485	9.23
160	3.714	0.363	9.978	0.076	0.197	2080	411	7.96
200	3.540	0.395	9.490	0.153	0.187	2385	447	8.42
Pc 1.02								
50	0.955	0.212	104.724	76.121	0.122	284,830	34,761	276
80	0.907	0.235	331.286	75.972	0.129	288,357	37,324	265
120	0.935	0.198	510.865	63.577	0.119	264,859	31,514	254
160	1.357	0.176	859.301	41.557	0.090	254,135	22,900	338
200	1.339	0.166	1368.857	34.861	0.090	298,485	26,876	394

[a] r = 0.953–1.000 (biomass); r = 0.858–0.998 (bacteriocin).

Type 1

Strain Lb 2.01 (Fig. 2) represented typically the behavior of the most numerous group (Lb 2.01; Lb 3.03; Lb 3.04; and, less markedly, Lc 1.04) of all the groups tested. This group, which is summarized in Fig. 5, may be characterized by the fact that the production of not only biomass, but also of bacteriocins and lactic acid (the four strains displayed homofermentative metabolism) tended to increase toward both extremes of the oxygen availability interval; the opposite was true with carbohydrate consumption and, less markedly, pH value at the time of maximum biomass.

In the typical case of Lb 2.01, the profiles of biomass, bacteriocin, lactic acid, pH, and carbohydrate consumption according to time were grouped into two levels: one with high production and high consumption levels, which corresponded to the maximum and minimum loads, and another with low production and consumption, which corresponded to the three intermediate loads, with little difference among these three. In addition, under the extreme conditions, it was particularly evident that the production of bacteriocin continued after the biomass reached the asymptotic value; this did not occur under intermediate conditions. This suggests that bacteriocin can change its metabolic character (primary or secondary in the Luedeking and Piret sense) in response to the conditions of aeration. In the least typical case of the group (Lc 1.04, with one of the highest bacteriocin productions; see Table 2), the load scarcely established differences in this respect. This may be attributed to the scarce biomass production inherent in this strain, because under these circumstances, diffusion limits, which depend on the load and the viscosity of the medium (affected by the biomass), are not sufficient to establish important differences in the percentage of oxygen saturation.

Although it constitutes an empirical fact that we have verified repeatedly, this tendency toward an increase in production toward the two extremes of availability of oxygen is difficult to justify. A possible hypothesis would imply accepting that under conditions of intense aerobiosis or near restriction of oxygen the microorganism maintains an enzymatic group directed toward a particular metabolic mode, while under intermediate conditions it may be compelled to synthesize the necessary enzymes for both types of pathways. Although we have not investigated this problem, it may be noted that the hypothesis suggests several possible methods of verification, among these the quantification of the levels of key enzymes, or the energetic state of the microorganism by means of the relationship between adenine nucleotides in the different situations.

Type 2

Type 2 behavior appeared characteristically with Pc 1.02 and Cb 1.01 (the latter is not a bacteriocin-producing strain, but basically it may be assimilated to this behavior). In the typical case (Figs. 3 and 5D), the production of biomass, bacteriocin, and lactic acid increased as the availability

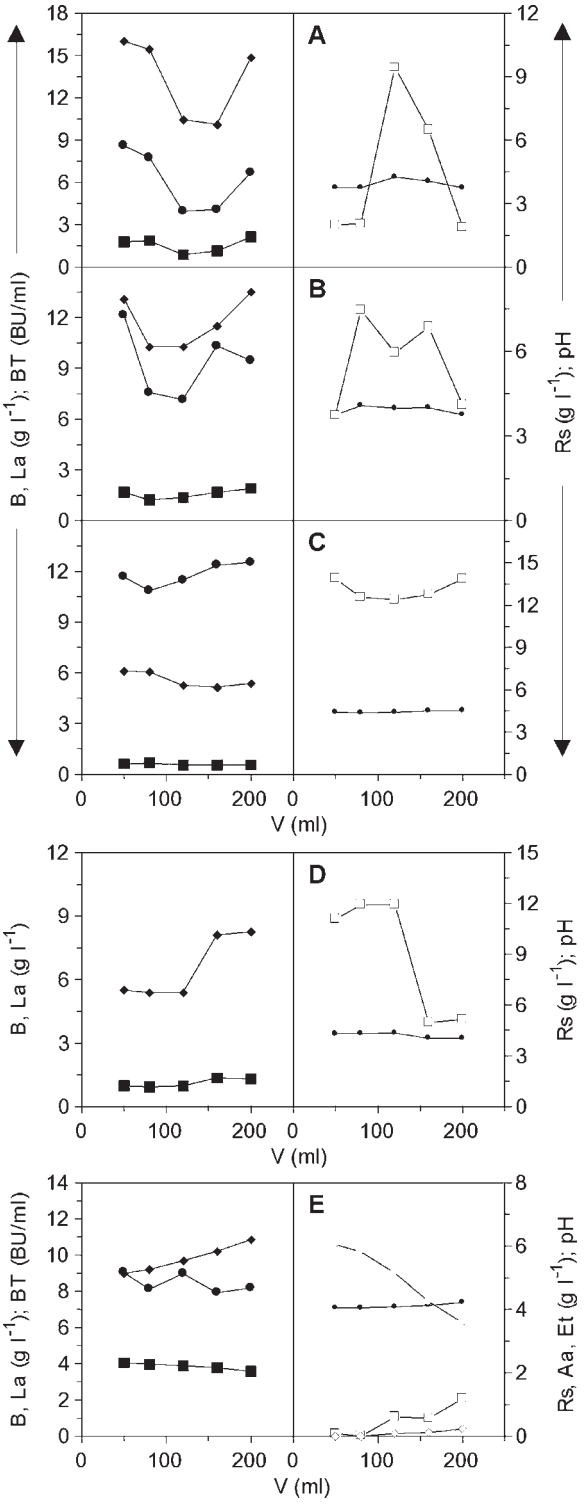

Fig. 5. Effect of flask load (V) on (•) pH, (■) biomass, (●) bacteriocin, (◆) lactic acid, (□) reducing sugars, (○) acetic acid, and (◇) ethanol on corresponding cases. **(A)** Lb 3.03; **(B)** Lb 3.04; **(C)** Lc 1.04; **(D)** Cb 1.01; **(E)** Ln 3.07. Other symbolic notations are as in Fig. 4.

of oxygen diminished, while the metabolic character of the bacteriocin (pediocin) remained primary under all aeration conditions.

Type 3

Finally, with Lb 6.04 and Ln 3.07 (Figs. 4 and 5E), production increased with the availability of oxygen, and the bacteriocin presented a primary metabolite character under all conditions. In these cases, which displayed a clearly heterofermentative behavior, with the appearance of lactic acid, acetic acid, and ethanol, the availability of oxygen affected also the relationship between the final metabolites in both strains, which prevents their direct comparison with the preceding groups. In both cases, in fact, the production of lactic acid and ethanol diminished as oxygen availability increased, whereas acetic acid behaved in the opposite way.

A more unified description (Fig. 6) can be based on the NADH + H^+ consumptions calculated from the final levels of the lactic metabolites detected, and considering the stoichiometry of the pathways described by Cocaign-Bousquet et al. *(11)*. Such a description, however, does not alter our preceding classification. In homofermentative cases, the results are, logically, redundant to those of Figs. 2 and 3. In heterofermentative cases, that could suggest some aeration-dependent trends, taking into account the levels of some metabolites, the NADH + H^+ balance still clearly demonstrates the scarce or null effect of this variable, with the possible exception of Lb 6.04, where it is possible to appreciate a slight tendency toward the increase of reducing necessities with the descent of the degree of aeration.

Quantification of Response in Terms of Kinetic Parameters

The growth of lactic acid bacteria and the production of bacteriocins have been described by means of different mathematical models *(6,10,12–15)*, among which the logistic model, simple and generalized, is suitable for all the experimental profiles found (*see* Figs. 2–4) and allows calculation of parameters with a definite biologic meaning, useful for comparative purposes. To do this, the following considerations were used as a starting point.

First, the differential equation that describes the growth rate is as follows:

$$r_B = \frac{dB}{dt} = \mu_m B \left(\frac{K - B}{K}\right) \tag{1}$$

from which, by integration between $B_0 \rightarrow B$ and $0 \rightarrow t$, the explicit expression that describes the production of biomass according to time (logistic) is obtained:

$$B = \frac{K}{1 + e^{c - \mu_m t}} \tag{2}$$

in which $c = \ln[(K/B_0) - 1]$.

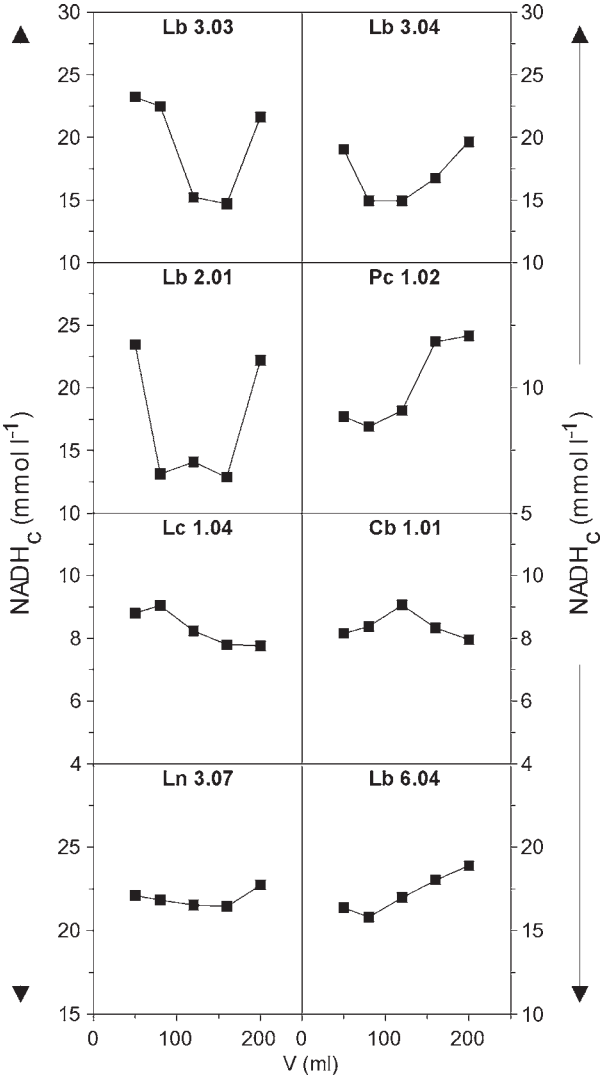

Fig. 6. Maximum NADH + H^+ consumptions (mmol/L) calculated from final levels of lactic metabolites, considering stoichiometry of pathways described by Cocaign-Bousquet et al. *(11)*.

Second, the rate of bacteriocin production r_P can be described by means of the classic model of Luedeking and Piret *(16)*:

$$r_p = \frac{dP}{dt} = \alpha\frac{dB}{dt} + \beta B = \alpha r_B + \beta B \quad (3)$$

which is commonly expressed by dividing both terms by biomass; hence,

$$\frac{r_P}{B} = \alpha\frac{r_B}{B} + \beta \text{ and } \frac{r_P}{B} = \alpha\mu + \beta \quad (4)$$

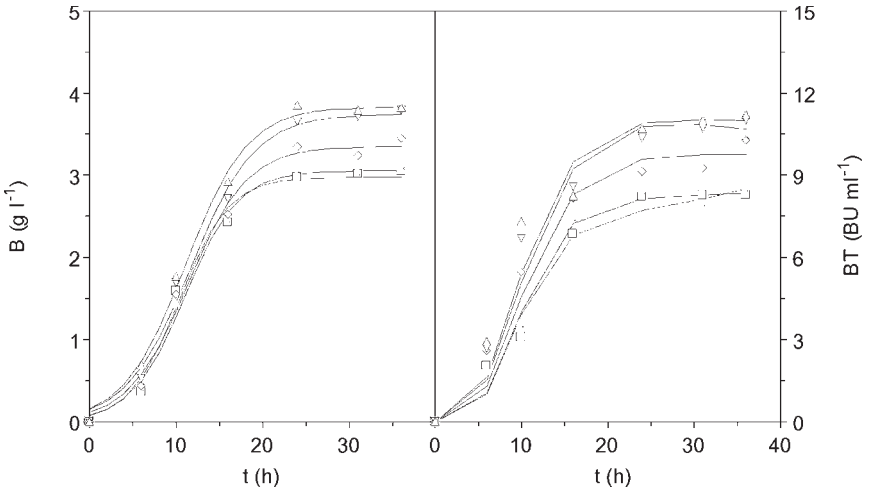

Fig. 7. Experimental data (dotted lines) corresponding to time courses of biomass (B) and bacteriocin (BT) in *L. helveticus* cultures shown in Fig. 4, and fits (solid lines) to logistic (Eq. 5; left) and Luedeking and Piret (Eq. 6; right) equations.

This formulation enables microbial metabolites to be classified as primary (production rate dependent on rate of biomass production: $\beta = 0$), secondary (production rate dependent on biomass present: $\alpha = 0$), and mixed (production rate simultaneously dependent on growth rate and biomass present: $\alpha \neq 0$ and $\beta \neq 0$).

Third, the numerical integration of rate r_B provides the actual biomass B_R. From the substitution of r_B and B_R in the Luedeking and Piret equation, the actual rate of bacteriocin production, r_P, can be obtained:

$$B_R = \sum_{t=0}^{t=t} r_B \tag{5}$$

$$BT_R = \sum_{t=0}^{t=t} r_P = \sum_{t=0}^{t=t} (\alpha r_B + \beta B_R) \tag{6}$$

Figure 7 shows the experimental results and the corresponding adjustments to Eqs. 5 and 6 for cultures of Lb 6.04 at all load volumes assayed. The example is representative of all of the strains studied, which in all cases produced linear correlation coefficients between observed and model-predicted values within the interval 0.858–1.000. Table 2 provides the corresponding estimations of the parameters and yields defined in the Nomenclature.

Acknowledgments

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Nomenclature

B = biomass (g/L)
B_0 = initial biomass (g/L)
B_R = rate of actual biomass production (g/[L·h])
BT_m = maximum bacteriocin obtained from culture (BU/mL)
BT_R = rate of actual bacteriocin production (BU/[mL·h])
BU = bacteriocin arbitrary units
K = maximum biomass (g/L)
r_B = growth rate (g/[L·h])
r_p = production rate for product P (bacteriocin) (BU/[mL·h])
$Y_{B/G}$ = biomass production/glucose consumption (g of biomass/g of glucose)
$Y_{BT/B}$ = bacteriocin production/biomass production (BU/g of biomass)
$Y_{BT/G}$ = bacteriocin production/glucose consumption (BU/g of glucose)
α = Luedeking and Piret parameter (to be experimentally determined) (BU/[10^3·g])
β = Luedeking and Piret parameter (to be experimentally determined) (BU/[10^3·g·h])
μ_m = specific maximum growth rate (biomass production per unit of biomass and time) (h^{-1})

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