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 due to the facultative anaerobic character of these micro-organisms. Studying the cultures of eight strains of lactic acid bacteria carried out in vessels with different loads of medium, within an interval which determines linearly the minimum availability of oxygen, the existence of three types of behaviour was highlighted: production increases (a) with the availability of oxygen, (b) with the restriction of this availability and (c) towards both extremes of these conditions, diminishing in intermediate situations. These behaviours not only affected the production of bacteriocins, but also their metabolic character (in the Luedeking and Piret sense), likewise the production of other characteristic metabolites, such as lactic acid, acetic acid and ethanol.

1 INTRODUCTION

Lactic acid bacteria are facultative anaerobic micro-organisms, i.e. capable of modifying a central part of their metabolic pathways 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, the lactic acid appears accompanied, in aerobiosis, by different proportions of several oxidised 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 [15, 16], 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, 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 bibliography, contributes. In fact, although cultures are often carried out disregarding control or measurement of the oxygen level, the abovementioned 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. Thus, for example, while Hirsch [7], De Vuyst [5] or Kim *et al* [8], 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* [3], find, with the same strains, maximum production under clearly aerobic conditions.

In order to deal with this problem, this study compared the time-course of the most characteristic descriptive variables of the cultures of these micro-organisms, in eight strains of lactic acid bacteria (*Lactobacillus brevis*, *Lactobacillus casei* (two strains), *Lactobacillus helveticus*, *Lactococcus lactis*, *Leuconostoc mesenteroides*, *Pediococcus acidilactici* and *Carnobacterium piscicola*), growing under different aeration conditions. Said conditions were obtained by resorting to 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.

2 MATERIALS AND METHODS

2.1 Microbiological methods

The strains used, together with their sources, are shown in Table 1. Stock cultures were stored at – 50°C in powdered skimmed milk suspension with 25% glycerol [2]. Inocula (1% v/v) consisted of cellular suspensions from 12-24 hour-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 orbital shaking. All assays were carried out in triplicate.

2.2 Analytical methods

At pre-established times, each culture sample was divided into two aliquots. The first was centrifuged at 5,000 rpm for 15 min, and the sediment washed twice and resuspended in distilled water to the adequate dilution for measuring the absorbance at 700 nm. The dry weight can then be estimated from a previous calibration curve for each strains. The corresponding supernatant was used for the determination of reducing sugars [1], proteins [12], and lactic acid, acetic acid and ethanol by HPLC analysis (refractive-index detector), using a ION-300 column (Interaction Chromatography, USA) with 6mM sulphuric acid as a mobile phase (flow=0.4 ml.min⁻¹) at 65°C. The second aliquot was used for the extraction and quantification of bacteriocin, according to methods described in detail in previous works [2, 10], using *Carnobacterium 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.

2.3 Numerical methods

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

3 RESULTS AND DISCUSSION

3.1 Types of response to the degree of aeration

As was to be expected, the loads of the recipients clearly conditioned the time-course of the oxygen saturation percentages (pO₂), 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 pO₂ minimum for the three strains representative of the three behaviours defined below, and which 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 containers 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 which they represent, according to the load in the containers recipients, the consumption of nutrients and the characteristic yield of lactic acid bacteria measured at the time of maximum biomass. Taking into account the totality of the information shown in figures 2, 3 and 4 (representative of all the group studied, whose characterisation in terms of kinetic parameters is shown in Table 3), it is possible to define three basic types of response to the degree of aerobiosis.

Type 1. The strain Lb 2.01 (figure 2) represented typically the behaviour of the most numerous group (Lb 2.01, Lb 3.03, Lb 3.04, and, less markedly, Lc 1.04) of the whole tested. Said group, that is summarized in the superior part of the figure 5, may be characterised 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 towards 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, in fact, 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 between these three. Besides, under the extreme conditions, it was particularly evident that the production of bacteriocin continues after the biomass has reached the asymptotic value; this does 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 3), the load scarcely establishes differences in this respect. This may be attributed to the scarce biomass production inherent in this strain, as 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 which we have verified repeatedly, this tendency towards an increase in production towards 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 micro-organism maintains an enzymatic group directed towards a particular metabolic mode, while under intermediate conditions it may be compelled to synthesise 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 micro-organism by means of the relationship between adenine nucleotides in the different situations.

Type 2. This type appeared characteristically with Pc 1.02 and Cb 1.01 (the latter is not a bacteriocin-producing strains, but basically it may be assimilated to this behaviour). In the typical case (figure 3 and figure 5, center), the production of biomass, bacteriocin and lactic acid increased as the availability of oxygen diminished, while the metabolic character of the bacteriocin (pediocin) remained primary under all aeration conditions.

Type 3. Finally, with Lb 6.04 (figure 4 and figure 5, down) and Ln 3.07, 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 behaviour, with the appearance of lactic acid, acetic acid and ethanol, it could also be noted that the availability of oxygen affected likewise 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, while acetic acid behaved in the opposite way.

A more unified description (figure 6) can be based on the $\text{NADH}+\text{H}^+$ consumptions calculated from the final levels of the lactic metabolites detected, and considering the stoichiometry of the pathways described by Coccagn-Bousquet [4]. Such a description, however, does not alter our preceding classification. In homofermentative cases the results are, logically, redundant with those of the figures 2 and 3. In heterofermentative cases, that –on the basis of the levels of some metabolites– could suggest some aeration-dependent trends, the $\text{NADH}+\text{H}^+$ balance still leaves clearer the scarce or null effect of this variable, maybe with the exception of Lb 6.04, where it is possible to appreciate a slight tendency to the increase of reducing necessities with the descent of the aeration degree.

3.2 Quantification of the 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 [3, 6, 9, 11, 14], among which the logistic model, simple and generalised, is suitable for all the experimental profiles found (see figures 2, 3 and 4), and allows calculation of parameters with a definite biological meaning, useful for comparative purposes. To do this, the following considerations were used as a starting point:

1: The differential equation which describes the growth rate is:

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

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

$$B = \frac{K}{1 + e^{c - \mu_m t}}; \text{ where } c = \ln \left(\frac{K}{B_0} - 1 \right) \quad (2)$$

2: The rate of bacteriocin production r_P can be described by means of the classical model of Luedeking and Piret [13]:

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

commonly expressed by dividing both terms by biomass, so:

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

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 $\beta \neq 0$).

3: 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:

$$B_R = \sum_{t=0}^{t=t} r_B \quad (5)$$

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

Figure 7 shows the experimental results and the corresponding adjustments to equations (5) and (6) for cultures of Lb 6.04 at all load volumes assayed. The example is representative of the whole 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. The corresponding estimations of the parameters and yields defined in Table 2 are shown in Table 3.

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TABLE 1: Strains used.

Strains	Reference	Abbreviated key
<i>Lactobacillus brevis</i>	CECT 216	Lb 2.01
<i>Lactobacillus casei ssp. casei</i>	CECT 4040	Lb 3.03
<i>Lactobacillus casei ssp. casei</i>	CECT 4043	Lb 3.04
<i>Lactobacillus helveticus</i>	CECT 541	Lb 6.04
<i>Lactococcus lactis ssp. lactis</i>	CECT 539	Lc 1.04
<i>Leuconostoc mesenteroides ssp. mesenteroides</i>	CECT 4046	Ln 3.07
<i>Pediococcus acidilactici</i>	NRRL B-5627	Pc 1.02
<i>Carnobacterium piscicola</i>	CECT 4020	Cb 1.01

CECT: Spanish Type Culture Collection (*Colección Española de Cultivos Tipo*).

NRRL: Northern Regional Research Laboratory (Peoria, Illinois, USA).

TABLE 2: Symbolic notations used. BU: Bacteriocin arbitrary units.

B :	Biomass. Dimensions : g.l^{-1}
K :	Maximum biomass. Dimensions: g.l^{-1}
μ_m :	Specific maximum growth rate (biomass production per unit of biomass and time). Dimensions: h^{-1}
B_0 :	Initial biomass. Dimensions: g.l^{-1}
α :	Luedeking and Piret parameter (to be experimentally determined). Dimensions: $\text{BU.10}^{-3}.\text{g}^{-1}$
β :	Luedeking and Piret parameter (to be experimentally determined). Dimensions: $\text{BU.10}^{-3}.\text{g}^{-1}.\text{h}^{-1}$
r_B :	Growth rate. Dimensions : $\text{g.l}^{-1}.\text{h}^{-1}$
r_p :	Production rate for product P (bacteriocin). Dimensions: $\text{BU.ml}^{-1}.\text{h}^{-1}$
B_R :	Rate of actual biomass production. Dimensions: $\text{g.l}^{-1}.\text{h}^{-1}$
BT_R :	Rate of actual bacteriocin production. Dimensions: $\text{BU.ml}^{-1}.\text{h}^{-1}$
BT_m :	Maximum bacteriocin obtained from culture. Dimensions: BU.ml^{-1}
$Y_{B/G}$:	Biomass production / glucose consumption: $\text{g biomass / g glucose}$
$Y_{BT/B}$:	Bacteriocin production / biomass production: BU / g biomass
$Y_{BT/G}$:	Bacteriocin production / glucose consumption: BU / g glucose

TABLE 3: Main parametric estimations (as defined in Table 2) of kinetic models (5) and (6) describing biomass and bacteriocin production by lactic acid bacteria growing in cultures with different load volumes. $r=0.953-1.000$ (biomass); $r=0.858-0.998$ (bacteriocin)

	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	17969	2064	12.54
80	0.641	0.982	71.352	0.636	0.101	17103	1731	11.76
120	0.567	1.417	71.314	0.748	0.082	18737	1543	11.48
160	0.563	1.559	75.983	1.087	0.080	21679	1744	12.37
200	0.572	1.052	83.654	0.944	0.094	22105	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	284830	34761	276
80	0.907	0.235	331.286	75.972	0.129	288357	37324	265
120	0.935	0.198	510.865	63.577	0.119	264859	31514	254
160	1.357	0.176	859.301	41.557	0.090	254135	22900	338
200	1.339	0.166	1368.857	34.861	0.090	298485	26876	394

FIGURE CAPTIONS

Figure 1: Relationships between flask load and minimum pO_2 in the three strains ($\square$: Lb 2.01, $\circ$: Pc 1.02, $\diamond$: Lb 6.04) representative of the three types of response to oxygen availability. Experimental values (dotted lines) and fits (continuous lines) to simple linear models.

Figure 2: Above and centre: time-course of *L. brevis* cultures at different flask loads ($\square$: 200, $\circ$: 160, $\diamond$: 120, ∇ : 80 and $\triangle$: 50 ml). B: biomass; BT: bacteriocin; Rs: reducing sugars; Pr: proteins; La: lactic acid. Below: effect of flask load (V) on pH ($\bullet$), biomass ($\blacksquare$) bacteriocin ($\bullet$), lactic acid ($\blacklozenge$) and consumption of reducing sugars ($\square$).

Figure 3: Above and centre: time-course of *P. acidilactici* cultures at different flask loads ($\square$: 200, $\circ$: 160, $\diamond$: 120, ∇ : 80 and $\triangle$: 50 ml). B: biomass; BT: bacteriocin; Rs: reducing sugars; Pr: proteins; La: lactic acid. Below: effect of flask load (V) on pH ($\bullet$), biomass ($\blacksquare$) bacteriocin ($\bullet$), lactic acid ($\blacklozenge$) and consumption of reducing sugars ($\square$).

Figure 4: Above and centre: time-course of *L. helveticus* cultures at different flask loads ($\square$: 200, $\circ$: 160, $\diamond$: 120, ∇ : 80 and $\triangle$: 50 ml). B: biomass; BT: bacteriocin; Rs: reducing sugars; Pr: proteins; La: lactic acid; Aa: acetic acid; Et: ethanol. Below: effect of flask load (V) on pH ($\bullet$), biomass ($\blacksquare$) bacteriocin ($\bullet$), lactic acid ($\blacklozenge$), acetic acid ($\circ$), ethanol ($\diamond$) and consumption of reducing sugars ($\square$).

Figure 5: Effect of flask load (V) on pH ($\bullet$), biomass ($\blacksquare$) bacteriocin ($\bullet$), lactic acid ($\blacklozenge$), reducing sugars ($\square$) and acetic acid ($\circ$) and ethanol ($\diamond$) on the correspondent cases. A: Lb 3.03; B: Lb 3.04; C: Lc 1.04; D: Cb 1.01; E: Ln 3.07. The rest of the symbolic notations as in figure 4.

Figure 6: Maximum $\text{NADH}+\text{H}^+$ consumptions (in mmol l^{-1}) calculated from the final levels of the lactic metabolites, considering the stoichiometry of the pathways described by Coccagn-Bousquet et al. (2002).

Figure 7: Experimental data (dotted lines) corresponding to the time-courses of biomass and bacteriocin in *L. helveticus* cultures shown in figure 4, and fits (continuous lines) to the logistic ((5), left) and Luedeking and Piret ((6), right) equations.

FIGURE 1

FIGURE 2

FIGURE 3

FIGURE 4

FIGURE 5

FIGURE 7

FIGURE 8