Substrate inhibition of *Pediococcus acidilactici* by glucose on a waste medium. Simulations and experimental results

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ABSTRACT

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Aims: The possibility of substrate inhibition by glucose on biomass and pediocin production was studied in cultures of *Pediococcus acidilactici* on a residual medium.

Methods and Results: Calculation of the substrate inhibition coefficient in the context of microbial growth is generally laborious, and very prone to experimental error. However, a simulation combining logistic and Monod kinetics equations demonstrates that quantitative evidence for this type of inhibition, without the possibility of misinterpretation, can be obtained through the comparison of punctual preasymptotic productions as a function of substrate concentration.

Significance and Impact of the Study: It was concluded that glucose had an inhibitory effect on growth, but not on bacteriocin production.

Keywords: pediocin production, *Pediococcus acidilactici*, substrate inhibition.

INTRODUCTION

Bacteriocins, peptides with antimicrobial activity produced by lactic acid (LAB) and other groups of bacteria, are of great interest to the food industry as they are innocuous, sensitive to digestive proteases, and do not change the organoleptic properties of the food. In view of the relatively high cost of commercial media recommended for LAB culture (Daba *et al.* 1993; De Vuyst 1995), we have evaluated residual substrates such as milk whey or mussel processing wastes (MPW) as substitutes (Amiali *et al.* 1998; Goulhen *et al.* 1999; Guerra and Pastrana 2001). In this respect, MPW, which are a widely available and easily saccharificable starchy medium with moderate protein content (González *et al.* 1992; Murado *et al.* 1993; Pastrana *et al.* 1995; Pintado *et al.* 1999), could represent an appropriate growth substrate.

In the evaluation of a microbial culture medium it is important to be able to define quickly the kinetic of growth.

By use of multivariable factorial analysis, Guerra and Pastrana (2002) were able to predict the inhibition of the pediocin production by *Pediococcus acidilactici* by the presence of glucose in MPW. This was not predicted by the classical and more laborious approach to the Monod model. In this paper we report a new procedure for evaluating potential microbial growth substrates that combines the flexibility of the factorial methods with the robustness of the classical approach. The developed method is mathematically robust, experimentally verified and can be generalized to any type of microbial culture.

MATERIALS AND METHODS

Micro-organisms and culture media

Pediococcus acidilactici NRRL B-5627 a pediocin-producing strain, was provided by the Northern Regional Research Laboratory (Peoria, IL, USA). The basic culture medium (M) was prepared by saccharification and dilution of MPW by methods described previously (González *et al.* 1992; Murado *et al.* 1993; Pastrana *et al.* 1995), and included the following (g l^{-1}): glucose (5·0); proteins (0·3); total

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phosphorus (0·06). Starting from M medium, the following were also prepared:

Mg media: M medium supplemented with various concentrations of glucose (10, 20, 40 and 60 g l^{-1}).

MgP1 and MgP2 media: Mg media supplemented with peptone (6 and 12 g l^{-1}, respectively).

MgY media: Mg media supplemented with yeast extract (6 g l^{-1}).

In all cases, pH was adjusted to 7·0 and solutions were sterilized by steam flow for 60 min. Cultures were carried out in 250–ml Erlenmeyer flasks with 50 ml of medium, at 30°C, with orbital shaking at 200 rev min^{-1}. Inocula (1% v/v) consisted of cellular suspensions from 20-h-aged cultures on M medium, adjusted to an O.D. (700 nm) of 0·90.

Analytical methods

At pre-established times, each culture sample was divided into two aliquots. The first was centrifuged at 10 000 *g* for 15 min, and the sediment washed twice and resuspended in distilled water to an appropriate dilution to measure O.D. at 700 nm. The dry weight can then be estimated from a previous calibration curve. The corresponding supernatant was used for the determination (data not showed) of reducing sugars, proteins and lactic acid. The second aliquot was used for the extraction and quantification of bacteriocin, using *Carnobacterium piscicola* CECT 4020 (Spanish Type Culture Collection) as an indicator, according to the method of Cabo *et al.* (1999). All assays were carried out in duplicate.

Numerical methods

Fitting procedures and parametric estimations calculated from the results, as much in simulations as in the inhibition experiments, 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.

RESULTS AND DISCUSSION

Simulation of microbial growth with and without substrate inhibition

The calculation of the substrate inhibition coefficient in the context of microbial growth generally requires a laborious experimentation and is sensitive to error. However, quantitative evidence for this type of inhibition can be reduced by comparing production, prior to reaching their asymptotic values, as a function of substrate concentration. In addition, it can be demonstrated that although this methodology can

raise doubts about its validity, it even leads to results without possibility of misinterpretation.

In effect, it is conventionally assumed that the growth of a microbial culture can be described by means of the logistic equation:

$$B = \frac{K}{1 + \exp(c - \mu t)} \quad (1)$$

where $c = \ln [(K/B_0)-1]$; B, biomass (B_0, initial biomass); K, maximum biomass; μ, specific growth rate.

In addition, the specific maximum (initial) growth rate depends on the initial substrate concentration in accordance with the Monod equation:

$$\mu = \frac{\mu_m S}{k_m + S + k_i S^2}, \quad (2)$$

where S, substrate concentration; μ, specific growth rate, with μ_m as maximum value; k_m, Monod constant; k_i, substrate inhibition coefficient ($k_i = 0$, without substrate inhibition).

Thus, substituting μ, in eqn 1, for its value in eqn 2

$$B = \frac{K}{1 + \exp \left(c - \frac{\mu_m S}{k_m + S + k_i S^2} t \right)} \quad (3)$$

$k_i = 0$, without substrate inhibition.

Accordingly, using arbitrary values for μ_m, k_m and k_i, eqn 3 allows us to simulate a series of growth curves (Fig. 1, centre) with specific growth rates depending on the initial substrate concentration (S) (Fig. 1, upper). Bisecting these curves with a straight line parallel to the ordinate axis (biomass) to a value of the abscissa (time) previous to the asymptote, a series of preasymptotic values of biomass can be derived as a function of S. Such values produce an asymptotic profile, if $k_i = 0$, and a profile decreasing after a maximum, if $k_i \neq 0$.

A more realistic assumption is to accept that the initial substrate concentration affects not only the specific rate μ, but also the maximum biomass K of the logistic process. In this case, it is sufficient to introduce the term $K = bS$ into eqn 3, where b is a constant of proportionality:

$$B = \frac{bS}{1 + \exp \left(c - \frac{\mu_m S}{k_m + S + k_i S^2} t \right)} \quad (4)$$

$k_i = 0$, without substrate inhibition.

Under the same conditions of the previous case this expression gives the curve grouping in Fig. 2. Moreover, as shown in the lower parts of Figs 1 and 2, the resulting profiles can be approximately adjusted to equations of the form:

$$B = \frac{B_m^{ap} S}{k_m^{ap} + S + k_i^{ap} S^2} \quad (5)$$

$k_i^{ap} = 0$, without substrate inhibition where the superscript ap indicates apparent values of the corresponding magnitudes.
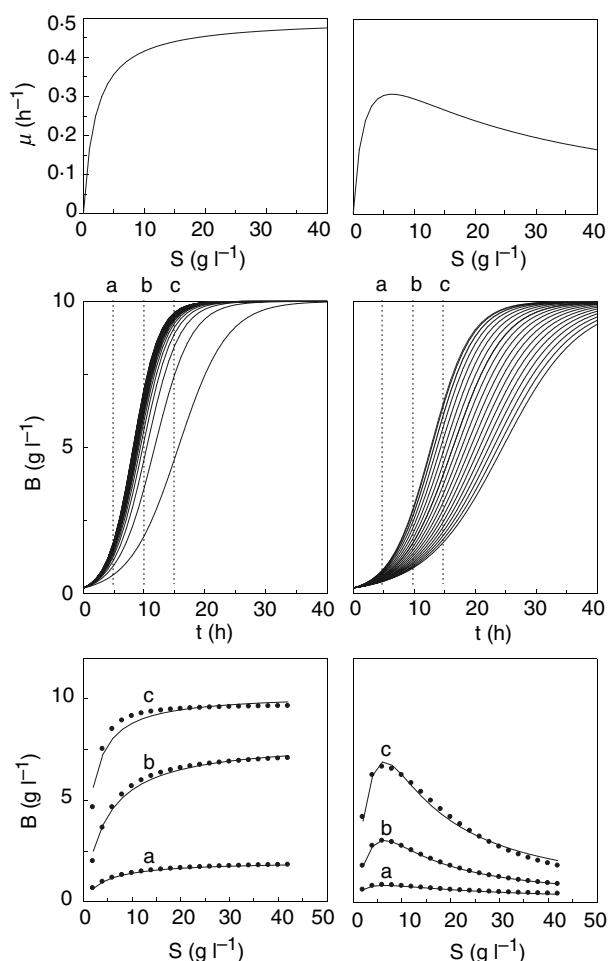
Fig. 1 Upper: Monod equations with (right) and without (left) substrate inhibition. Centre: logistical growth curves obtained at different initial substrate concentrations, assuming that this variable only affects specific growth rate μ. Lower: substrate–biomass relationships at three preasymptotic times (*a, b, c*). Continuous line shows the fitting of the points to eqn 5. Left: $k_i^{ap} = 0$; right: $k_i^{ap} \neq 0$

Fig. 2 As Fig. 1, assuming that initial substrate concentration affects the specific growth rate μ and maximum biomass K

The usefulness and limitations of these models will be discussed in the section of conclusions.

Substrate inhibition in *P. acidilactici* cultures

Figure 3 shows the results obtained when the conditions described above are applied to cultures of *P. acidilactici* at two preasymptotic incubation periods (16 and 22 h) in different media with the specified glucose supplements. The parametric estimations calculated from eqn 5 for biomass and pediocin at 16 h are given in Table 1.

These figures and tables illustrate that the kinetic behaviour of the biomass and pediocin productions are affected by the glucose, peptone and yeast extract
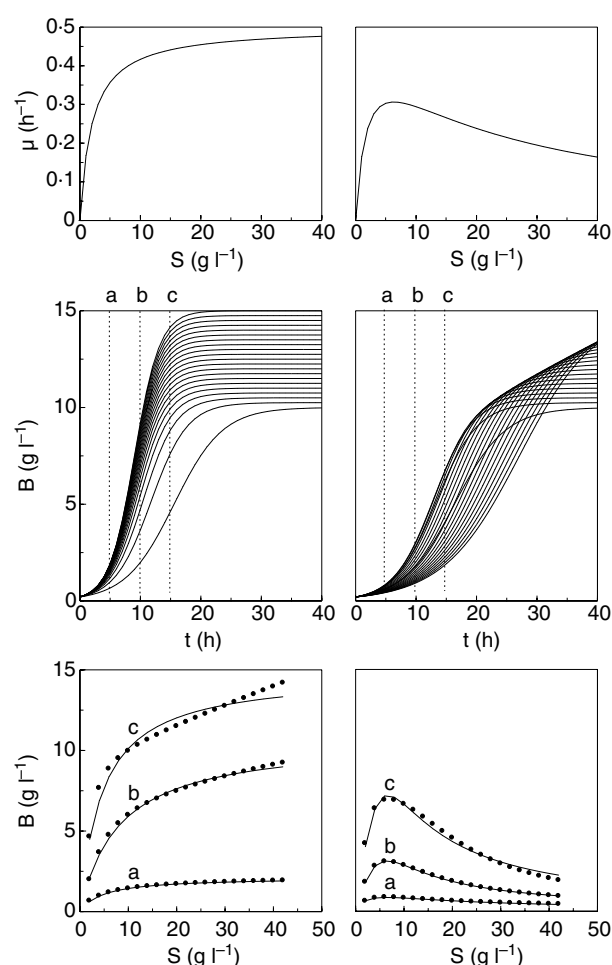
supplements. With regard to biomass, glucose causes a susbtrate inhibitory effect under all conditions and incubation times ($0{\cdot}010 \geq k_i^{ap} \geq 0{\cdot}006$), while stimulatory effect of yeast extract is located between the ones due to the two assayed peptone levels. Pediocin production is not sensitive to the inhibition by glucose, or the effect is scarcely detectable ($0{\cdot}002 \geq K_i^{ap} \geq 0$), while the stimulatory effect of peptone, even at the lowest tested level, is larger than from yeast extract.

CONCLUSIONS

An important step in the evaluation of a waste material as component of a microbial culture medium, is the study of the potential inhibitory effects of some of the substrates on growth or the production of metabolites. The method proposed here has the interest of allowing a quantitative treatment of these issues even in those (frequent) cases in
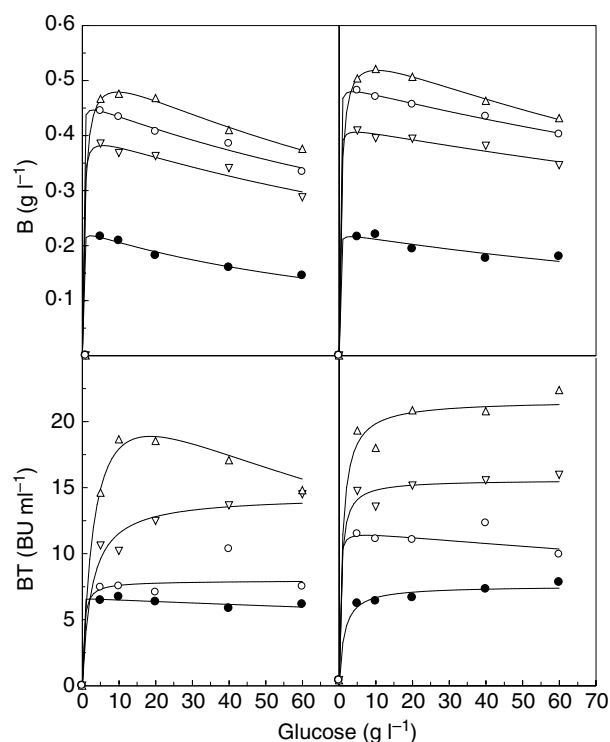
Fig. 3 Effects of initial glucose concentration on biomass (*B*) and bacteriocin (BT) production by *Pediococcus acidilactici* at incubation times of 16 (left) and 22 h (right), on different media: Mg (●), MgP1 (▽), MgP2 (△), MgL (○)

Table 1 Apparent parameters of eq 5 when applied to the relationship between biomass (*B*) or bacteriocin (BT) and initial glucose concentration in cultures of *Pediococcus acidilactici* on different media after incubation period (16 h)

16 h	Mg	MgP1	MgP2	MgY
B_m^{ap} (g l^{-1})	0·228	0·408	0·550	0·462
k_m^{ap} (g l^{-1})	0·050	0·191	0·711	0·050
k_i^{ap} (l g^{-1})	0·010	0·006	0·008	0·006
r^2(*)	0·998	0·996	0·998	0·998
BT_m^{ap} (BU ml^{-1})	6·620	14·395	25·770	7·915
K_m^{ap} (g l^{-1})	0·009	2·472	3·375	0·413
K_i^{ap} (l g^{-1})	0·002	0	0·001	0
r^2(*)	0·994	0·976	0·996	0·966

*Correlation coefficient between expected and experimental values.

which it is carried out as a too schematic experimentation, which cannot be used to apply the more prolix conventional approach by means of Monod equation. However, the validity of our method is not surprising, as the biomass produced within a certain time interval is a function of the corresponding production rate.

The lack of curve fitting that is observed in the Figs 1 and 2 is clearly because of the use of a hyperbolic function for describing values derived from a combination of hyperbolic and exponential functions. In view of the fact that such rates vary nonlinearly with time, it is also clear that the values of the parameters B_m^{ap}, k_m^{ap} and k_i^{ap} (BT_m^{ap}, K_m^{ap}, K_i^{ap}, for bacteriocin inhibition) are only apparent, i.e. they are functions of the real parameters of the Monod equation. However, the presence of inhibition substrates can be identified unequivocally by making rigorous quantitative comparisons between series of cultures carried out under the same conditions.

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