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Peptones from autohydrolised fish viscera for nisin and pediocin production.

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Abstract

Various peptones obtained from hydrolysed visceral homogenates of four fishery residues showed their suitability for promoting the growth of lactic acid bacteria, micro-organisms with particularly complex requirements regarding peptidic nutrients. The assay of several treatments with two bacterial species, producers of the two main bacteriocins (nisin and pediocin) demostrated that optimum conditions only imply a brief autohydrolysis at natural pH and room temperature, with subsequent steam-flow stabilisation. Later kinetic analysis of the cultures of both bacteria in the best media provided parameters which, for production of both biomass and bacteriocins (the latter behaved in the majority of cases as a secondary metabolite), indicate comparable or superior results to those found in costly commercial media, specifically recommended for culture of lactic acid bacteria.

1. Introduction

One problem associated with the production of lactic acid bacteria and bacteriocins on an industrial scale (especially for applications based on their probiotic effects) is their demand for diversified peptidic sources, which several commercial media (MRS, TGE, APT) resolve by including products such as bactopeptone, triptone, meat extract or yeast extract (sometimes all of these) in formulations which reach high prices. However, in the absence of special culture resources, such as the use of stepped pH profiles, (Cabo et al., 2001a), the efficiency (substrate consumed/initial substrate) of these media is usually low, suggesting unbalanced proportions of nutrients. Thus, the peptidic materials which remain in the media at the end of the productive period constitute superfluous expenditure and hinder the subsequent purification of the bacteriocins obtained. The inorganic sources of nitrogen do not produce acceptable results (Guerra and Pastrana, 2001), nor the –apparently obvious– solution of adjusting the initial concentrations of the protein sources to the detected consumption is suitable (Cabo et al., 2001b). This is so because the peptones do not represent only a source of organic nitrogen, but rather a source of amino acids or specific peptides, therefore only a fraction of the quantities added is relevant.

Although in some cases the need for certain amino acids admits concrete justifications (such as contributing to the lantionine ring, in lantibiotic bacteriocins), studies in this respect do not totally explain the highly superior effects of complex protein sources (*e.g.* Kozak et al., 1977; De Vuyst, 1995). Thus, attention was directed towards peptides (Biswas et al, 1991; Parente and Hill, 1992), to which two types of role have been attributed. First, it has been shown that certain peptides (structurally similar to bacteriocins, or not; generated, or not, by the same bacteria) induce the biosynthesis of bacteriocins, entering into the cell and acting as activating signals for transcription, or –by means of a "secondary messenger" mechanism– activating a

transmembrane protein from outside the cell, which produces the effective signal in the intracellular space. In the biosynthesis of nisin by *Lactococcus lactis* a self-induced secondary messenger mechanism acts, being the nisin excreted which activates the transmembrane protein (Kuipers et al., 1995). The activating peptide excreted by *Lactobacillus sake* is only similar to bacteriocin (Eijsink et al., 1996).

Other authors impute a less specific role (precursor or energetic) to peptides, and point out that the systems of transport through the membrane of lactic acid bacteria are more efficient with peptides than with free amino acids (Monnet and Gripon, 1994); or that the amino acids are protected from the catabolic reactions in the peptidic structures (Law, 1983; Payne, 1980); or that the intracellular hydrolysis of the peptides produces an energy which economizes carbohydrates during growth (Aasen et al., 2000). It has also been suggested that efficacy in these respects requires a peptidic size of between 4 and 14 amino acids (Matthews and Payne, 1975), though attempts to obtain more concrete definitions have not led to conclusive results.

These functions, especially the second, make it reasonable to suppose that if an intestinal space can maintain a stable consortium of lactic acid bacteria –as numerous evidence suggests–, a relevant factor in the formation of the consortium is the composition of the peptidic fractions produced by proteolytic enzymes of the digestive tract. Thus, the autohydrolysis of viscera homogenates could generate peptidic fragments (peptones) suitable for the culture of lactic acid bacteria. While commercial peptones are derived principally from casein, soya, gelatine and meat, peptones from fish have already given good results in applications such as the production of proteases by *Bacillus subtilis* (Ellouz et al., 2001), of gastrine and epidermal growth factor (EGF) by mouse fibroblasts (Cancre et al., 1999), of glycerol by *Saccharomyces cerevisiae* (Kurbanoglu and Kurbanoglu, 2003), and promoting good microbial growth in other cases (De La Broise et al., 1998; Duffosé et al., 2001). On the other hand, on coasts such as that of Galicia

(NW of Spain), numerous industrial facilities dedicated to the transformation of fishing or aquaculture products generate extensive availability of reject viscera, currently wasted, and suitable for this use.

Based on these considerations, this study examines the suitability for the culture of lactic acid bacteria of media formulated with autohydrolysates of fish viscera obtained from different species and work conditions. The micro-organisms tested were two lactic acid bacteria, producers of the two most important bacteriocins: nisin (*Lactococcus lactis*) and pediocin (*Pediococcus acidilactici*). Two media were used as terms of comparison: commercial MRS (usual for the culture of lactic acid bacteria) and another in which the fish peptone was replaced by commercial bactopeptone. As criteria for comparison, in a first screening, the biomass and bacteriocin production for two incubation times were used. In a second set of tests with the most productive media, the assessment was based on the kinetic parameters of the cultures, obtained by numerical adjustment of the results to two mathematical models widely applied to this type of descriptions.

2. Materials and methods

2.1. Preparation of fish autohydrolysates

Raw materials, provided by different fish processing companies, were viscera from squid (*Loligo vulgaris*), yellowfin tuna (*Thunnus albacares*), swordfish (*Xiphias gladius*), and rainbow trout (*Oncorhynchus mykiss*), sampled immediately after industrial processing and stored for a maximum period of 15 days at –20ºC until its use.

The viscera masses were grinded with ~10% (v/w) of distilled water, the homogenate of each species being divided into the 20 aliquots necessary to obtain the peptones corresponding to 4 pH values and 5 incubation times. The pH values used were that of each homogenate (all of them between 6.16 and 6.39) and three others, obtained by the addition of the necessary quantities of 4N HCl to obtain three diminishing values in steps of approximately 1 unit of pH; the lowest values being between 3.12 and 3.40. The autohydrolyses were carried out maintaining the homogenates at 20ºC, with orbital shaking at 100 rpm, for periods of 0, 8, 18, 30 and 48 hours.

After each incubation period, the autohydrolysates were stabilised by steam flow (101ºC/1 hour) and were treated in a centrifuge decanter at 6000 rpm for 15 minutes (small volumes may be paper-filtered in a Büchner), to obtain the corresponding sediments (potentially useful as substrate in biological silage) and supernatants. The supernatants (or fish peptones) were typified determining the levels of total nitrogen, remaining protein, leucine and tyrosine released, and total sugars, and then stored at –20ºC until the time of their use in the formulation of culture media. The basic composition of peptones at natural pH, with the hydrolysis degree corresponding to time 0, is shown in Table 1.

2.2. Microbiological methods

The micro-organisms used were *Lactococcus lactis* CECT 539 (abbreviated key Lc 1.04) from Spanish Type Culture Collection, and *Pediococcus acidilactici* NRRL B-5627 (Pc 1.02), kindly provided by the Northern Regional Research Laboratory (Peoria, Illinois, USA). *Carnobacterium piscicola* CECT 4020 was used as an indicator for bacteriocin bioassay. Stock cultures were stored at –50ºC in powdered skimmed milk suspension with 25% glycerol (Cabo et al., 2001b). Inocula (1% vol/vol) consisted of cellular suspensions from 12- (Lc 1.04) and 24- (Pc 1.02) hour aged cultures in MRS medium, adjusted to an OD (700 nm) of 0.900.

The composition of the media is shown in Table 2. For comparative purposes, a medium (BP) was used where the fish peptones were replaced by a commercial bactopeptone solution with an equivalent protein level (Lowry), as well as an MRS commercial medium. In all cases, initial pH was adjusted to 7.0 and solutions were sterilised at 121ºC for 15 min. Micro-organisms were grown in 300 ml Erlenmeyer flasks with 200 (Pc 1.02) or 100 ml (Lc 1.04) of medium at 30ºC, with 200 rpm orbital shaking. The cultures were carried out in triplicate. At pre-established times, each culture was divided into two aliquots. The first was centrifuged at 5,000 rpm for 15 min, and the sediment washed twice and re-suspended in distilled water to the adequate dilution to measure the absorbance at 700 nm. The dry weight can then be estimated from a previous calibration curve. The corresponding supernatant was used for the determination of reducing sugars (Bernfeld, 1951), proteins, glucose, lactic acid and acetic acid. The second aliquot was used for the extraction and quantification of bacteriocin. All assays were carried out in duplicate.

2.3. Analytical methods

The proteins were determined in all matrices (hydrolysates and supernatants of cultures) by the method of Lowry et al. (1951). In the case of the hydrolysates, total nitrogen (Havilah et al., 1977, applied to digests obtained by the classic procedure of Kjeldahl), tyrosine (Barker and Worgan method, 1981), leucine (Sarath et al., 1989) and total sugars (Dubois et al., 1956 according to the application of Strickland and Parsons, 1968) were also determined. In the case of the supernatants, glucose, lactic acid and acetic acid were quantified by HPLC analysis (refractive-index detector), using an ION-300 column (Interaction Chromatography, USA) with 6mM sulphuric acid as a mobile phase (flow=0.4 $ml.min^{-1}$), at 65ºC. Methods for the extraction and quantification of bacteriocin were described in detail by Cabo et al. (1999) and Murado et al. (2002), using *Carnobacterium piscicola* CECT 4020 as an indicator.

2.4. Numerical methods

Fitting procedures and parametric estimations calculated from the 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. Autohydrolysis of viscera homogenates

The method described for the obtaining of hydrolysates leads in all cases to the recovery of 0.7-0.8 litres of peptonated solution per kg (fresh weight) of processed viscera, with no statistically significant differences due to the species. The progress of the hydrolysis slightly increases the volume recovered, in equivalent proportions in all species (up to 10-15% after 48 hours).

Figure 1 shows the progress of the different autohydrolysis processes, quantified via the release of two indicative amino acids (leucine and tyrosine). It is obvious that such indicators (likewise the soluble protein, not shown due to being redundant) are not suitable for showing possible relevant changes in the peptidic compositions from the nutritional point of view. However, their variations through time could detect the existence of differentiated phases in the corresponding processes, potentially useful for establishing borders. This was not so; even if there were occasional differences derived from the species, the amino acid considered and the initial pH value (which remains approximately constant throughout hydrolysis), in general terms, all the

processes showed basically asymptotic tendencies during the period studied, without any irregularities which might imply important changes in the mechanisms of the hydrolysis. It may be added that according to released leucine –which seems to be a more discriminative indicator than tyrosine–, the highest initial pH (*i.e.* that of the unmodified homogenate) is generally the most favourable for hydrolysis.

3.2. L. lactis and P. acidilactici cultures on fish peptones

Samples of the cultures, the kinetics of which conform to the logistic model, were taken at times corresponding to the logarithmic phase and the beginning of the asymptotic phase (8 and 16 hours for Lc 1.04; 20 and 40 hours for Pc 1.02). Although both groups of samples led to mutually coherent results, in tables 3 to 6 only the values obtained at the start of the asymptotic phase are compared, these being more representative of the productions studied. The regularities deduced from the results are as follows:

1: In the most productive cultures with fish peptones: (a) the biomasses surpassed those obtained with BP and MRS media. (b) The nisin always surpassed that obtained with BP and approximately equalled that obtained with MRS. (c) The pediocin always amply surpassed that obtained with BP and approximately equalled that obtained with MRS in the cases of trout and swordfish, was lower with squid and higher with yellowfin tuna. Furthermore, the maximum productions of biomass and bacteriocins corresponded to the same conditions of hydrolysis, except in the case of nisin with yellowfin tuna, where a slight disparity was noticed.

2: The highest productions of biomasses and bacteriocins with fish peptones were obtained at the lowest hydrolysis time, with two exceptions: the biomass of Lc 1.04 with yellowfin tuna hydrolysate and pediocin with squid hydrolysate, both with their maximums at the maximum

hydrolysis time. On the other hand, the pH of the hydrolysis did not significantly affect the maximum productions (*i.e.* those corresponding to the minimum hydrolysis time), with the same exceptions: Lc 1.04-biomass-yellowfin tuna (maximum at pH=6.23) and pediocin-squid (maximum at pH=4.27).

The suitability of the low hydrolysis times is somewhat surprising, as initially a greater peptidic diversity and nutritional capacity for micro-organisms could have been expected with the more advanced hydrolysates. The result (repeatedly confirmed) may be interpreted by admitting the exact opposite, or supposing that hydrolysis affects peptides of interest which are free at the initial stage of the system or, finally, that during the process of hydrolysis biogenic amines are released (Enes Dapkevicius et al., 2000), which inhibit the growth of certain micro-organisms.

3.3. Cultures with selected fish peptones

In order to obtain a more detailed and formal description of microbial growths on fish peptone media, the 32 cultures (Table 7) resulting from the combination of the two bacteria tested with the four hydrolysates of each species which led to the best bacteriocin productions were subsequently carried out, using MRS medium as a term of comparison. As well as biomasses, bacteriocins and pH, in these cultures the levels of metabolites characteristic to lactic acid bacteria (lactic acid and acetic acid, the latter an indicator of heterofermentative metabolism) were determined, likewise the consumption of glucose, proteins, total nitrogen and total phosphorus. Figures 2 and 3 show the time-courses in the case of peptones from trout, which are representative of the joint of the results. As a whole, results are consistent with the assessment derived from the earlier screening, and may indicate the advisability of rejecting media SF2, SF3 and SF4 for nisin production, due to their prolonged lag phase (SF2) or low production (SF3 and SF4) promoted with Lc 1.04.

3.4. Kinetic models

There are many models applicable to the description of microbial growth and metabolite production, with the possibility of inputting terms useful in particular cases, such as those which translate dependences on temperature or pH (*e.g.* Rincon et al., 1993; Gadgil and Venkatesh, 1997; Åkerberg et al., 1998; Cabo et al., 2001a), or inhibition by substrate or product (*e.g.* Åkerberg et al., 1998; Hofvendahl et al., 1999; Kumar et al., 1996; Gonçalves et al., 1997; Cachon and Diviès, 1994; Callewaert and De Vuyst, 2000). Among the most generally used models which adapt to sigmoid profiles such as those in figures 2 and 3, we find the Gompertz and logistic equations.

The first, useful with data which present appreciable phases of latency (such as those of processes which require adaptation of the inoculum to the medium), is of a more complex structure than the second and, in its habitual form with microbiological applications (re-parameterised by Zwietering et al., 1990) resorts to the logarithmic normalisation:

$$\ln \frac{X}{X_0} = A \exp\left\{ - \exp\left[\frac{\mu_{\max}(\lambda - t)\exp(1)}{A} + 1 \right] \right\} \text{ ; where } X_m = X_0 \exp(A)$$

X biomass (g.l^{-1})
X_0 initial biomass (g.l^{-1})
X_m maximum biomass (g.l^{-1})
A maximum biomass at $t \to \infty$ (dimensionless)
t time (hours)
μ_{max} maximum specific increment (dimensions of t^{-1}) of the dependent variable [ln (X/X_0)]
λ lag phase (dimensions of t)

Although typically applied for describing logarithmic increases in relative cell number, an useful parameter in the comparisons proposed here is the maximum specific growth rate, the relationship of which with μ_{max} is complex and very sensitive to the experimental error in the value of X_0. Under these conditions we have preferred the logistic equation, easily managed, with parameters of clear meaning and pseudokinetic structure, already used with satisfactory results in combination with the Luedeking and Piret model (1959) and modifications which allow the input of other effects, to describe growth and production of bacteriocins in lactic acid bacteria cultures (Cabo et al., 2001a). To do this, we start from the following assumptions:

1: The production of biomass as a function of the time is given by:

$$X = \frac{K}{1 + e^{c - \mu_m t}} \;;\; \text{where } c = \ln\left(\frac{K}{X_0} - 1\right) \qquad [1]$$

2: The rate of bacteriocin production r_P can be described by means of the classical model of Luedeking and Piret (1959):

$$r_P = \alpha\, r_X + \beta X \qquad [2]$$

commonly expressed by dividing both terms by biomass, so:

$$\frac{r_P}{X} = \alpha \frac{r_X}{X} + \beta \quad ; \quad \text{and:} \qquad \frac{r_P}{X} = \alpha\mu + \beta \qquad [3]$$

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

on biomass present: α=0 and β≠0), and mixed (production rate simultaneously dependent on growth rate and biomass present: α≠0 and β≠0).

3: The numerical integration of rate r_X provides the actual biomass X_R. Substituting r_X and X_R in the Luedeking and Piret equation, the actual rate of bacteriocin production, r_P, can be obtained:

$$X_R = \sum_{t=0}^{t=t} r_X \qquad [4]$$

$$BT_R = \sum_{t=0}^{t=t} r_P = \sum_{t=0}^{t=t} \left(\alpha\ r_X + \beta X_R\right) \qquad [5]$$

Figures 4 and 5 show the experimental results and their adjustments to equations [4] and [5] for both bacteria in the media formulated with peptones from yellowfin tuna (cases representative of the whole, different from those in figures 2 and 3, are illustrated in this way). All productions led to satisfactory fittings, with linear correlation coefficients between expected and observed values within the intervals specified in Tables 8 and 9, where the estimations of the parameters and yields defined in the summary of symbolic notations (Table 10) are shown. It may be noted that both nisin and pediocin (of which it is known that they can behave as primary or secondary metabolites depending on the media and culture conditions) behave basically in these media as mixed metabolites (the fittings of the corresponding productions to the equation [5] are better when the restriction β=0 is omitted).

4. Conclusions

1: On coasts where industries dedicated to the transformation of fishing or aquaculture products are plentiful, the high proportions of viscera waste derived from that activity (and which represent nothing but an environmental problem) allow to obtain, in a simple and rapid way, autohydrolysates (from cephalopods, seawater and freshwater fishes) useful as microbiological culture media. This work demostrates that these hydrolysates can substitute other peptones in the habitual formulations for culture of lactic acid bacteria, promoting biomass and bacteriocin productions that equal or surpass those obtained on high-cost media recommended for these purposes. Previous results (Cabo et al., 2001b; Vázquez et al., 2003) showed that yeast extract levels as those used here only promote, in absence of peptones, a very weak growth of lactic acid bacteria. On the other hand, the suppression of yeast extract in fish peptone media led to results essentially equivalent to those shown in Tables 3, 4, 5 and 6.

2: In general, the highest productions of biomass and bacteriocins corresponded to the same conditions and were obtained at low hydrolysis times, the pH of the hydrolysis having a limited influence on the final levels. The preparation of fish peptones may thus be reduced to the homogenisation of the viscera mass with 10% of water (which promotes a sufficient degree of hydrolysis during the operating time at natural pH), stabilisation of the homogenate by steam flow and separation of the supernatant by filtration or centrifugal decantation.

3: The combined use of the logistic (biomass) and the Luedeking-Piret (bacteriocin) models allowed satisfactory adjustment of the microbial kinetics, providing values of predictive interest regarding the essential parameters of the cultures, which indicate the metabolically mixed character which may be expected of bacteriocin production in this type of media.

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TABLE 1: Main composition ($g.l^{-1}$) of peptones from fish viscera. YT: yellowfin tuna; SQ: squid; SF: sword fish; TR: rainbow trout.

	Proteins (Lowry)	Total sugars	Total nitrogen
YT	68.27	4.07	13.13
SQ	43.34	2.36	8.19
SF	54.02	2.26	11.06
TR	61.49	3.41	12.01

TABLE 2: Composition of culture media used in microbiological assays ($g.l^{-1}$).

	FP media [1]	BP medium	MRS medium
Glucose	20.00	20.00	20.00
Yeast extract	2.00	2.00	4.00
Sodium acetate	3.00	3.00	5.00
Ammonium citrate	2.00	2.00	2.00
K_2HPO_4	2.00	2.00	2.00
$MgSO_4$	0.20	0.20	0.20
$MnSO_4$	0.05	0.05	0.05
Tween 80	-	-	1.00
Meat extract	-	-	8.00
Bactopeptone	-	10.00	10.00
Fish peptone[1] protein (Lowry)	10.00	-	-

([1]): The four types of fish peptones defined in Table 1.

TABLE 3: Productions of biomass and bacteriocins on squid viscera hydrolysates, by cultures of *L. lactis* (Lc1.04) and *P. acidilactici* (Pc 1.02) at the beginning of the asymptotic phase.

Lc 1.04	**Hydrolysis time (h)**	**Initial pH of hydrolysis**			
		6.24	5.27	4.27	3.25
Biomass (g.l^{-1})	0	1.27	1.21	1.20	1.25
at 16 hours	8	0.79	0.72	0.74	0.72
	18	0.76	0.74	0.69	0.77
	30	0.76	0.68	0.70	0.81
	48	0.66	0.66	0.70	0.75
	MRS		0.82		
	BP		0.68		
Bacteriocin (BU.ml^{-1})	0	9.79	9.72	9.62	9.83
at 16 hours	8	9.72	9.74	9.66	8.89
	18	8.38	8.74	8.74	8.96
	30	9.71	8.73	8.20	8.99
	48	9.78	8.07	7.66	7.93
	MRS		10.13		
	BP		7.01		
Pc 1.02					
Biomass (g.l^{-1})	0	0.96	0.93	0.92	1.00
at 40 hours	8	0.72	0.72	1.05	0.93
	18	0.70	0.75	1.03	1.18
	30	0.71	0.77	0.94	1.11
	48	0.54	0.85	1.00	1.30
	MRS		1.31		
	BP		0.18		
Bacteriocin (BU.ml^{-1})	0	67.7	64.3	70.0	68.2
at 40 hours	8	62.0	95.0	81.1	59.5
	18	46.3	84.6	139.7	85.7
	30	71.1	91.8	126.6	98.2
	48	99.9	132.3	156.6	120.3
	MRS		223.6		
	BP		37.0		

TABLE 4: Productions of biomass and bacteriocins on trout viscera hydrolysates, by cultures of *L. lactis* (Lc1.04) and *P. acidilactici* (Pc 1.02) at the beginning of the asymptotic phase.

Lc 1.04	**Hydrolysis time (h)**	**Initial pH of hydrolysis**			
		6.16	5.17	4.13	3.12
Biomass (g.l^{-1}) at 16.5 hours	0	1.75	1.75	1.75	1.75
	8	1.55	1.17	1.14	1.06
	19	1.44	1.24	0.94	1.54
	29	1.24	1.22	1.27	1.11
	47	0.68	0.81	0.83	0.78
	MRS		0.82		
	BP		0.68		
Bacteriocin (BU.ml^{-1}) at 16.5 hours	0	7.31	7.31	7.31	7.31
	8	7.38	7.35	8.02	8.12
	19	7.36	8.50	8.54	7.96
	29	6.98	7.60	7.18	7.33
	47	6.41	7.45	8.01	7.74
	MRS		10.13		
	BP		7.01		
Pc 1.02					
Biomass (g.l^{-1}) at 40 hours	0	1.44	1.39	1.42	1.35
	8	1.25	1.58	1.31	1.43
	19	1.31	1.27	1.19	1.33
	29	1.31	1.37	1.26	1.44
	47	1.36	1.25	1.51	1.38
	MRS		1.31		
	BP		0.18		
Bacteriocin (BU.ml^{-1}) at 40 hours	0	179.1	178.3	181.2	185.2
	8	189.4	170.3	173.7	212.8
	19	137.2	104.9	109.3	112.0
	29	83.7	84.6	74.6	100.6
	47	125.6	127.2	74.3	113.3
	MRS		223.6		
	BP		37.0		

TABLE 5: Productions of biomass and bacteriocins on sword fish viscera hydrolysates, by cultures of *L. lactis* (Lc1.04) and *P. acidilactici* (Pc 1.02) at the beginning of the asymptotic phase.

Lc 1.04	**Hydrolysis time (h)**	**Initial pH of hydrolysis**			
		6.39	5.42	4.42	3.40
Biomass (g.l^{-1}) at 17 hours	0	1.28	1.21	1.16	1.10
	7	1.39	0.78	0.30	0.68
	19	1.01	0.42	0.50	0.94
	29	0.68	0.55	0.65	0.61
	47	0.74	0.53	0.85	0.88
	MRS		0.82		
	BP		0.68		
Bacteriocin (BU.ml^{-1}) at 17 hours	0	7.16	7.02	6.93	6.83
	7	6.81	5.82	3.47	3.72
	19	6.09	4.71	4.66	5.16
	29	5.80	5.39	4.81	4.04
	47	6.98	6.12	5.47	5.52
	MRS		10.13		
	BP		7.01		
Pc 1.02					
Biomass (g.l^{-1}) at 40 hours	0	1.48	1.40	1.35	1.36
	7	1.52	1.14	0.87	0.91
	19	1.16	0.94	0.80	0.89
	29	1.31	1.16	0.75	0.83
	47	1.25	0.92	0.92	0.98
	MRS		1.31		
	BP		0.18		
Bacteriocin (BU.ml^{-1}) at 40 hours	0	130.3	130.3	130.3	130.3
	7	110.9	113.7	103.1	111.3
	19	101.2	104.1	100.8	116.2
	29	110.0	107.9	86.8	74.8
	47	107.5	112.7	92.9	77.1
	MRS		223.6		
	BP		37.0		

TABLE 6: Productions of biomass and bacteriocins on yellowfin tuna viscera hydrolysates, by cultures of *L. lactis* (Lc1.04) and *P. acidilactici* (Pc 1.02) at the beginning of the asymptotic phase.

Lc 1.04	**Hydrolysis time (h)**	**Initial pH of hydrolysis**			
		6.23	5.33	4.35	3.29
Biomass (g.l^{-1}) at 16 hours	0	0.90	0.93	0.89	0.85
	8	0.95	0.97	1.09	1.24
	18	0.92	2.06	0.86	1.05
	30	1.24	2.08	1.22	1.33
	48	2.71	1.76	1.05	1.66
	MRS	0.82			
	BP	0.68			
Bacteriocin (BU.ml^{-1}) at 16 hours	0	9.20	8.82	9.15	9.20
	8	8.96	8.79	11.51	8.36
	18	3.19	2.00	8.86	9.84
	30	2.01	2.76	6.85	8.77
	48	0.00	10.48	8.99	7.08
	MRS	10.13			
	BP	7.01			
Pc 1.02					
Biomass (g.l^{-1}) at 40 hours	0	1.95	1.93	1.88	1.98
	8	1.25	1.64	1.77	1.71
	18	2.56	1.56	1.81	1.68
	30	2.13	1.41	1.70	1.80
	48	1.54	2.15	2.11	1.74
	MRS	1.31			
	BP	0.18			
Bacteriocin (BU.ml^{-1}) at 40 hours	0	349.6	347.2	352.1	341.3
	8	320.7	244.6	271.3	171.7
	18	217.7	142.9	178.7	198.1
	30	226.6	106.4	91.3	157.5
	48	181.8	271.5	211.3	245.7
	MRS	223.6			
	BP	37.0			

TABLE 7: Selected media for kinetic studies of Lc 1.04 and Pc 1.02 cultures					
		hydrolysis conditions for Lc 1.04		hydrolysis conditions for Pc 1.02	
fish viscera	key	time (h)	initial pH	time (h)	initial pH
Squid	SQ 1	0	6.24	18	4.27
	SQ 2	8	6.24	30	4.27
	SQ 3	8	5.27	48	5.27
	SQ 4	8	4.27	48	4.27
Trout	TR 1	19	5.17	0	6.16
	TR 2	19	4.13	8	6.16
	TR 3	19	3.12	8	4.13
	TR 4	19	4.13	8	3.12
Swordfish	SF 1	0	6.39	0	6.39
	SF 2	7	6.39	7	5.42
	SF 3	19	6.39	7	3.40
	SF 4	48	6.39	29	6.39
Tuna	YT 1	0	6.23	0	6.23
	YT 2	8	4.35	8	6.23
	YT 3	18	3.29	8	4.35
	YT 4	48	5.33	48	5.33

TABLE 8: Main parametric estimations (as defined in Table 10) from kinetic models [4] and [5] describing biomass and bacteriocin production by *L. lactis* on the specified media. r=0.988-0.999 (X); r=0.931-0.997 (BT)

key	K g l^{-1}	μ_m h^{-1}	α BU.10^{-3} g^{-1}	β BU.10^{-3} g^{-1}h^{-1}	$Y_{X/G}$ g X/g G	$Y_{BT/X}$ BU/g X	$Y_{BT/G}$ BU/g G	$Y_{BT/P}$ BU/g P	BT_m BU ml^{-1}
SQ 1	1.233	0.449	13.686	-	0.012	5308.9	613.1	28391.3	6.53
SQ 2	0.596	0.411	26.199	-	0.057	9573.8	545.8	18250.0	5.84
SQ 3	0.494	0.594	19.085	1.118	0.047	12166.9	571.4	68000.0	6.22
SQ 4	0.501	0.755	19.418	0.872	0.047	11346.5	536.0	19100.0	5.77
TR 1	1.565	0.675	8.815	0.003	0.138	4264.7	588.6	15950.0	6.38
TR 2	1.204	0.625	12.133	-	0.101	5752.7	578.9	19454.5	6.42
TR 3	1.516	1.449	5.911	0.608	0.130	5187.7	674.8	129000.0	7.74
TR 4	0.905	0.803	13.181	0.247	0.083	6801.3	562.8	103833.3	6.23
SF 1	1.380	0.422	16.445	-	0.139	4419.4	614.5	25120.0	7.19
SF 2	1.478	0.363	26.472	-	0.132	4924.9	650.6	8721.5	7.75
YT 1	0.745	0.880	12.898	0.646	0.078	10284.6	805.7	3702.4	7.59
YT 2	0.881	1.229	14.766	0.227	0.088	9712.9	856.3	3880.7	8.57
YT 3	0.763	1.176	16.365	0.104	0.075	9454.3	706.8	3634.1	7.47
YT 4	0.725	1.074	11.914	-	0.076	7001.3	530.4	2143.4	5.55
MRS	0.699	0.626	30.019	0.070	0.075	12549.8	952.5	56754.6	8.99

TABLE 9: Main parametric estimations (as defined in Table 10) from kinetic models [4] and [5] describing biomass and bacteriocin production by *P. acilactici* on the specified media. r=0.965-0.999 (X); r=0.973-0.997 (BT).

key	K g l^{-1}	μ_m h^{-1}	α BU.10^{-3} g^{-1}	β BU.10^{-3} g^{-1}h^{-1}	$Y_{X/G}$ g X/g G	$Y_{BT/X}$ BU/g X	$Y_{BT/G}$ BU/g G	$Y_{BT/P}$ BU/g P	BT_m BU ml^{-1}
SQ 1	0.885	0.139	741.102	8.047	0.081	150107.5	12160.3	178974.4	139.60
SQ 2	0.886	0.108	911.112	5.058	0.089	149156.3	13300.9	517785.7	144.98
SQ 3	0.851	0.120	889.537	1.087	0.080	132423.6	10639.7	160178.1	121.80
SQ 4	0.829	0.145	779.797	7.106	0.084	139762.6	11807.3	294413.0	137.49
TR 1	1.696	0.096	809.148	-	0.136	109237.5	14894.4	231691.4	195.46
TR 2	1.214	0.148	818.400	3.726	0.100	145275.6	14550.5	256250.0	184.50
TR 3	1.068	0.156	396.989	15.116	0.095	132658.2	12606.3	140357.1	157.20
TR 4	1.031	0.192	332.663	9.872	0.087	159119.3	13864.1	619428.6	173.44
SF 1	1.501	0.251	297.445	6.834	0.120	88849.7	10673.3	668150.0	138.13
SF 2	1.498	0.135	523.576	1.452	0.122	93165.4	11338.9	595043.5	136.86
SF 3	1.054	0.216	733.679	4.276	0.088	139746.3	12284.7	409257.1	158.39
SF 4	1.974	0.165	422.357	-	0.153	63428.9	9719.6	485230.8	131.40
YT 1	1.459	0.201	947.162	8.925	0.130	181034.9	23567.8	215504.0	288.28
YT 2	1.541	0.225	1297.157	-	0.129	177630.4	22838.2	235252.0	326.96
YT 3	1.999	0.205	642.932	11.026	0.139	153985.5	21424.5	129576.4	328.21
YT 4	1.717	0.170	889.207	10.561	0.127	175565.6	22236.6	215158.6	324.58
MRS	1.203	0.146	646.397	14.565	0.112	175155.1	19512.3	328487.5	221.22

TABLE 10: Symbolic notations used. BU: Bacteriocin arbitrary units.

X:	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}
X_0:	Initial biomass. Dimensions: g.l^{-1}
α:	Luedeking and Piret parameter (to be experimentally determined). Dimensions: BU.10^{-3}.g^{-1}
β:	Luedeking and Piret parameter (to be experimentally determined). Dimensions: BU.10^{-3}.g^{-1}.h^{-1}
r_X :	Growth rate. Dimensions : g.l^{-1}.h^{-1}
r_p:	Production rate for product P (bacteriocin). Dimensions: BU.ml^{-1}.h^{-1}
X_R:	Rate of actual biomass production. Dimensions: g.l^{-1}.h^{-1}
BT_R:	Rate of actual bacteriocin production. Dimensions: BU.ml^{-1}.h^{-1}
BT_m:	Maximum bacteriocin obtained from culture. Dimensions: BU.ml^{-1}
$Y_{X/G}$	Biomass production / glucose consumption: g biomass / g glucose
$Y_{X/P}$	Biomass production / protein consumption: g biomass / g protein
$Y_{BT/X}$	Bacteriocin production / biomass production: BU / g biomass
$Y_{BT/G}$	Bacteriocin production / glucose consumption: BU / g glucose
$Y_{BT/P}$	Bacteriocin production / protein consumption: BU / g protein

Figure captions

Figure 1: Autohydrolysis of viscera homogenates from fish products at different initial pH values (pH~6: □, pH~5: ○, pH~4: ◇, pH~3: ▽), evaluated in terms of leucine (leu) and tyrosine (tyr) released.

Figure 2: Time-course of *L. lactis* cultures on media prepared with trout peptones as peptidic source (TR 1:○ , TR 2: □, TR3: ◇, TR 4: ▽. Table 7), and on commercial MRS medium (▲). X: biomass, BT: bacteriocin (nisin), G: glucose, LA: lactic acid, AA: acetic acid.

Figure 3: Time-course of *P. acidilactici* cultures. BT: bacteriocin (pediocin). The rest of the keys as in fig. 2.

Figure 4: Experimental results (points) and fits (continuous lines) of *L. lactis* cultures on media prepared with yellowfin tuna peptones as peptidic source (YT 1: ○, YT 2: □, YT3: ◇, YT 4: △. Table 7), and on commercial MRS medium (●). Left: Biomasses and fits to logistic equation. Right: Bacteriocin (nisin) productions and fits to Luedeking and Piret equation.

Figure 5: Experimental results (points) and fits (continuous lines) of *P. acidilactici* cultures on the same media used in fig. 4. BT: bacteriocin (pediocin) productions. The rest of the keys as in fig. 4.

FIGURE 1

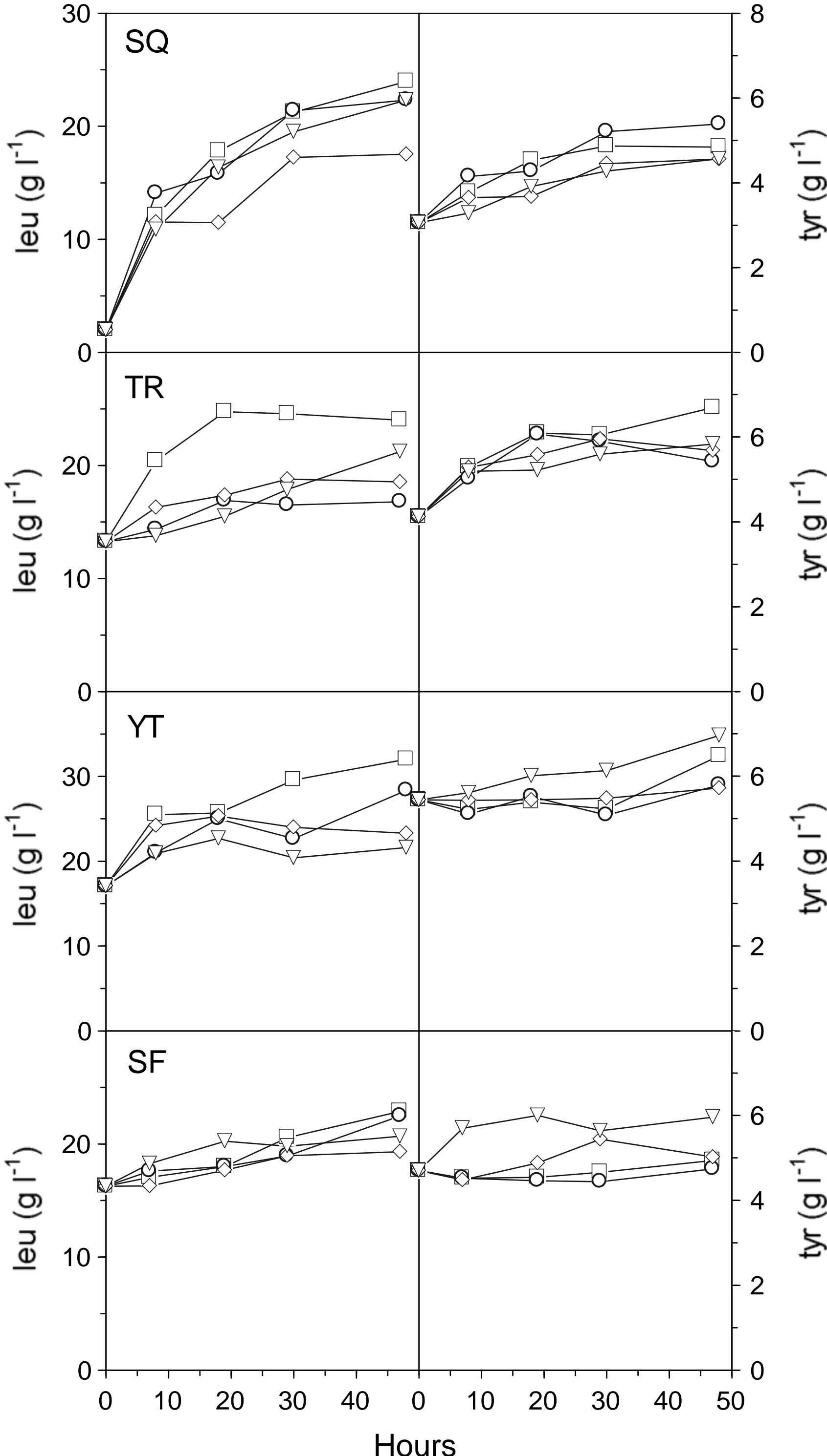

FIGURE 2

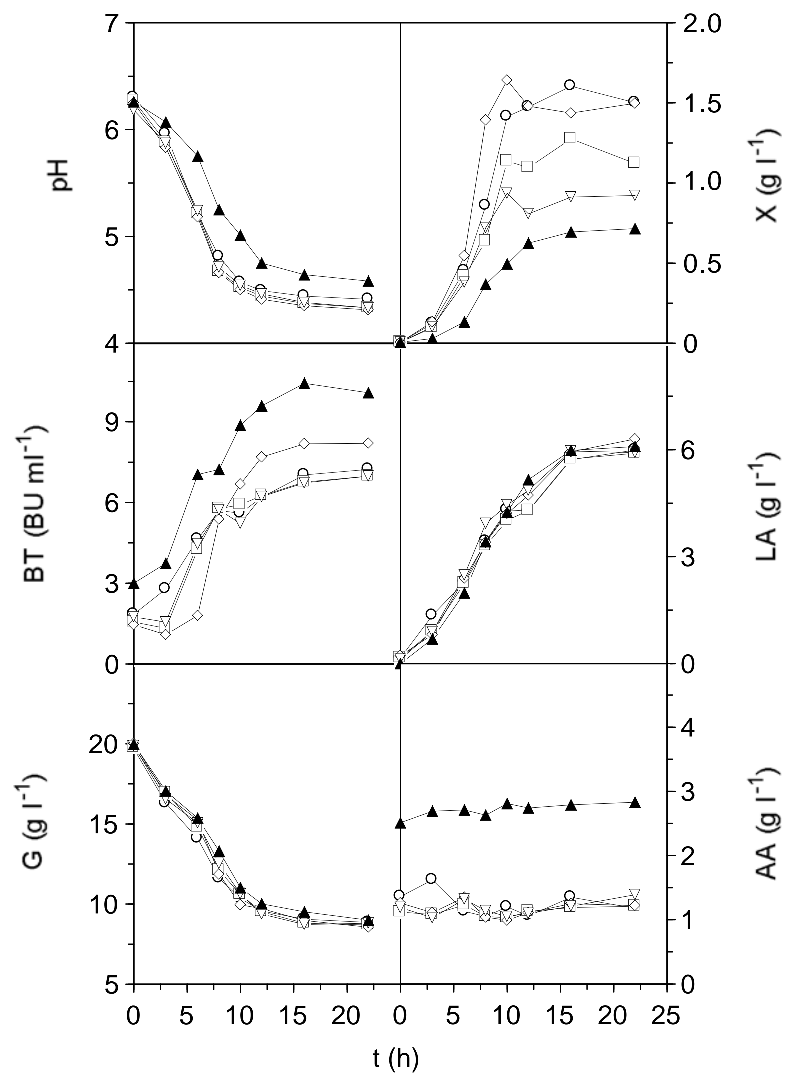

FIGURE 3

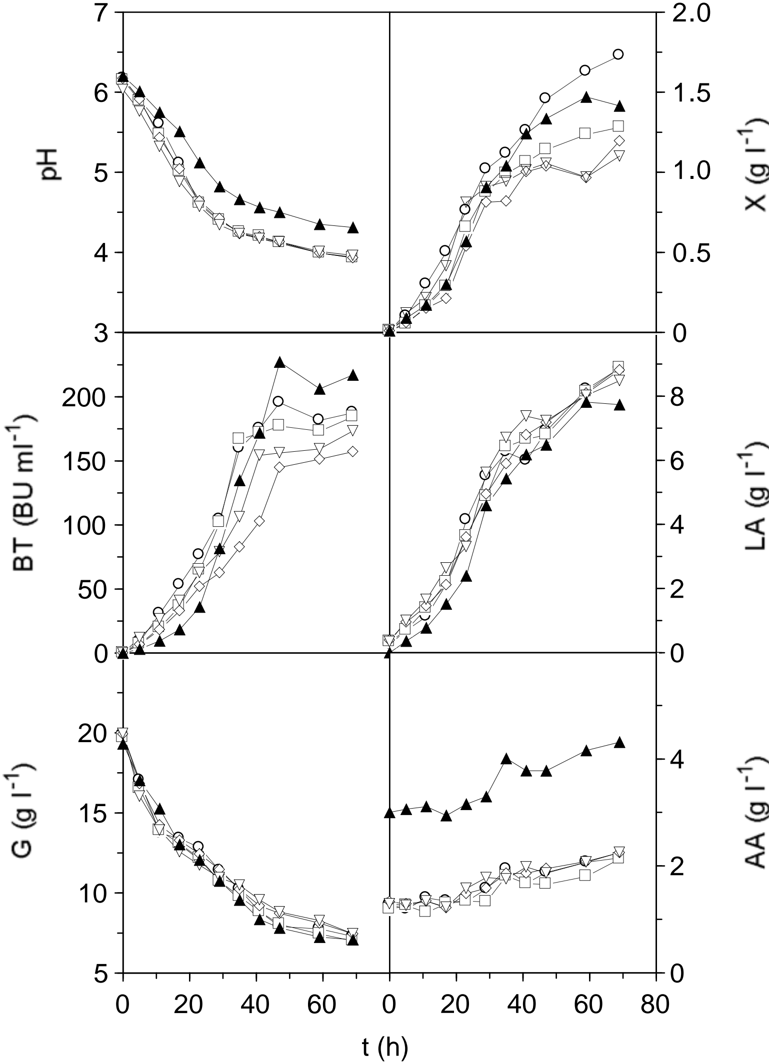

FIGURE 4