Enzymatic hydrolysates from food wastewater as a source of peptones for lactic acid bacteria productions.

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Headline: Food wastewater hydrolysates for lactic acid bacteria production.

Keywords: marine peptones; lactic acid bacteria production; nisin and pediocin production; lactic acid production; waste medium; logistic equation; waste valorisation.

ABSTRACT

A diverse group of peptones obtained by enzymatic hydrolysis of wastewater from the industrial processing of octopus showed their effectiveness to promote the growth of lactic acid bacteria and the bacteriocins production. The highest nisin formation by *Lactococcus lactis* was reached using peptones from papain hydrolysis for 24 hours (enzyme concentration: 1.25 mg papain/g protein). On the other hand, the highest pediocin production by *Pediococcus acidilactici* was obtained with peptones derived from 4 hour of pepsin digestion (enzyme concentration: 3.75 mg papain/g protein). Thus, these marine peptones are promising alternatives to currently available and expensive commercial medium as well as a possible solution to valorise this problematic wastewater.

Keywords: marine peptones; lactic acid bacteria production; nisin and pediocin production; lactic acid production; waste medium; logistic equation; waste valorisation.

INTRODUCTION

One of the most important environmental problems which are characteristic of coastline areas is the large volume of waste generated by fishing, aquaculture or foodstuff processing industries. Usually, these by-products are dumped into the sea without a previous treatment of depuration neither, evidently, valorisation. Among these food products, the cooked cephalopod (particularly octopus) is one of the higher commercial value and larger production of wastewater in the Galician coasts (NW Spain). These massive spills with high protein concentration generate a negative environmental impact on the *Galician Rías*, marine ecosystems of great ecological wealth and very sensitive to the contamination.

A possibility to eliminate this problem and to valorise those rich nitrogen and phosphorus organic sources (proteins, peptides and amino acids), is their use as nutrients in the formulation of optimum media for the microorganisms culture. To check their validity, the lactic acid bacteria (LAB) maybe the most appropriate group of microorganisms.

From the viewpoint of their industrial importance, LAB are classified as one of the greatest and most important microbial groups due to their significant role in food fermentation and preservation, as a natural microflora or as an inoculum added under controlled process conditions [1-3]. Among the molecules produced by these micro-organisms which present antimicrobial activity are lactic and acetic acid, ethanol, diacetyl, 2,3-butanediol and bacteriocins [4-6]. Bacteriocins produced by LAB, are peptides with antimicrobial activity and have great importance to the food industry as they are innocuous, sensitive to digestive proteases of vertebrates, and do not change the organoleptic properties of the food [7,8].

Lactic acid bacteria and, specifically, bacteriocins productions are very fastidious due to the need for rich growth media containing nutrients such as carbohydrates, nucleic acids, minerals, vitamins and, mainly, amino acids, proteins or protein hydrolysates [9-14]. For

example, the standard laboratory media (MRS, TGE, APT) solve the problem of protein sources, incorporating products such as bactopectone, tryptone, meat extract or yeast extract (sometimes all of these) on formulations which reach expensive costs. Also, these media are of bovine or porcine origin what disable their utility to generate *kosher* and *halal* products [11,15] and to avoid the bovine spongiform encephalopathy risk (BSE) [16].

It is hence that the use of low-cost proteins or protein fractions will bring about a reduction in large-scale production costs [10-13,17,18]. Furthermore, whether food waste is used to obtain these nutritional sources (e.g. waste generated by industries which process foodstuffs of marine origin), a complete productive cycle is closed: the recycling and valorisation of pollutant waste and the obtaining of a product of high added value, used for control and preservation of foodstuffs (LAB and bacteriocins).

Bearing this in mind, this work examined the suitability of media formulated with enzymatic hydrolysates of wastewater from the industrial processing of octopus, for the culture of LAB and the production of bacteriocins. The micro-organisms assayed were *Lactococcus lactis* and *Pediococcus acidilactici* producing of nisin and pediocin, respectively. The experimental approach consisted on kinetic studies of the protein fractions, using as criteria for comparison among the cultures, the parameters values obtained of the numeric adjustment of the experimental data to the proposed mathematical models.

MATERIALS AND METHODS

Preparation of marine peptone from octopus

The wastewaters from the industrial processing of octopus (*Octopus vulgaris*) were prepared in the laboratory, in a similar way to their commercial formulation, carrying out the following steps: the octopus was frozen immediately after capture and maintained at -50°C until its use, subsequently they were thawed in brine for 24 hours (brine solution: 20 g.l^{-1} of ClNa ; brine-to-octopus ratio: 500 ml per kg (dry basis)) and thermally processed in two separate stages at $90^{\circ}\text{C}/10\text{ min}$ in an autoclave. On average, the industrial processing of octopus generates approximately 0.75 litres of wastewater with high protein content per kilo (db) of material processed.

These octopus waters (12 l) were centrifuged at $7,500 \times g$ for 15 minutes at 20°C and divided into 24 aliquots of 500 ml each one, necessary to obtain the peptones corresponding to three types of enzymes, in two concentration levels and four incubation times. Papain (PAP; Sigma: 3.2 U/mg solid), trypsin (TR; Fluka: $\sim 9000\text{ U/mg}$) and pepsin (PEP; Merck: 700 FIP-U/g) were used as enzymes at the following levels and initial pH values:

- a) PAP: 1.25 mg enzyme/g protein aliquot, 12.50 mg enzyme/g protein aliquot, $\text{pH}=6.4$.
- b) TR: 1.25 mg enzyme/g protein aliquot, 3.75 mg enzyme/g protein aliquot, $\text{pH}=6.4$.
- c) PEP: 1.25 mg enzyme/g protein aliquot, 3.75 mg enzyme/g protein aliquot, $\text{pH}=2.5$.

Hydrolyses were carried out maintaining at 20°C with orbital shaking at 100 rpm for 0, 4, 10 or 24 hours and, subsequently, the proteolytic process was ended by heating to 101°C for 10 min. After each incubation period, the hydrolysates were treated in a decanting centrifuge at $7,500 \times g$ for 15 min., obtaining the corresponding sediment and supernatants. The supernatants (or marine peptones) were typified determining the levels of total nitrogen, soluble protein and total sugars, and then stored at -20°C until the time of their use in the

formulation of culture media. The basic composition of peptones with the hydrolysis degree corresponding to initial time was (in g.l⁻¹): 20.3 of protein (Lowry), 0.22 of reducing sugars, 3.7 of total nitrogen and 1.67 of total sugars.

Microbiological methods

The micro-organisms used were *Lactococcus lactis* subsp. *lactis* (abbreviated key Lc HD1) [8,19] and *Pediococcus acidilactici* NRRL B-5627 (Pc 1.02) [9,12]. *Leuconostoc mesenteroides* ssp. *lysis* (kindly donated by Dr. Ray, University of Wyoming, Laramie, USA) and *Carnobacterium piscicola* CECT 4020 (Spanish Collection Type Culture) were employed as indicators for the nisin and pediocin bioassays, respectively. Stock cultures were stored at -75°C MRS medium with 25% glycerol [20]. Inocula (1% vol/vol) consisted of cellular suspensions from 12 (Lc HD1) and 24 (Pc 1.02) hours aged in MRS, adjusted to an OD (700 nm) of 0.900.

Culture media

The composition of the media is shown in Table 1. For the purpose of comparison a MRS commercial medium (Pronadisa, Hispanlab S.A., Spain) was used. In all cases, initial pH was adjusted to 7.0 with NaOH 5M and solutions sterilised at 101°C for 1 hour. Micro-organisms were grown in 300 ml Erlenmeyer flasks with 200 ml of medium at 30°C, with 200 rpm orbital shaking. The cultures were carried out in duplicate. At pre-established times, each culture was divided into two aliquots. The first was centrifuged at 4,000 × g for 15 min, and the sediment washed twice and resuspended in distilled water to the adequate dilution for measuring the optical density (OD) at 700 nm. The dry weight can then be estimated from a previous calibration curve. The supernatant was used for the measure of reducing sugars [21], proteins, glucose, lactic acid and acetic acid. The second aliquot was

used for the extraction and quantification of bacteriocin (nisin and pediocin). All assays were carried out in triplicate.

Analytical methods

The proteins were determined by the method of Lowry et al. [22]. In the case of the hydrolysates, total nitrogen (method of Havilah et al. [23], applied to digests obtained by the classic procedure of Kjeldahl), and total sugars (Dubois et al. [24] according to the application of Strickland and Parsons [25]) 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 (Transgenomic, USA) with 6 mM sulphuric acid as a mobile phase (flow=0.4 ml.min⁻¹), at 65°C. Methods for the extraction and quantification of bacteriocin were described in detail by Cabo et al. [20] and Murado et al. [26,27], using *Leuconostoc mesenteroides* ssp. *lysis* (for nisin) and *Carnobacterium piscicola* CECT 4020 (for pediocin) as indicators.

Mathematical models

The mathematical models used to describe and to typify kinetically the sigmoid growth of microorganisms and the corresponding lactic acid bacteria productions were the following ones [28] (see symbol notation table 2 for the definition of the parameters and their units):

$$X = \frac{K}{1 + \exp\left[2 + \frac{4 \cdot v_{mx}}{K} \cdot (\lambda_x - t)\right]} \quad (1)$$

$$L = \frac{L_m}{1 + \exp \left[2 + \frac{4 \cdot v_{ml}}{L_m} \cdot (\lambda_l - t) \right]} \quad (2)$$

$$BT = \frac{BT_m}{1 + \exp \left[2 + \frac{4 \cdot v_{mb}}{BT_m} \cdot (\lambda_b - t) \right]} \quad (3)$$

An additional typification involved the calculation of the yields in the formation of biomass (X), lactic acid (L) and bacteriocins (BT) referred to both the consumption of proteins and of reducing sugars in all of the media and the two species assayed, this being assessed (see table of notations) in the following terms:

$$Y_{P/AR} = \frac{\Delta P}{\Delta AR} = \frac{P_f - P_i}{AR_i - AR_f} \quad (4)$$

$$Y_{P/Pr} = \frac{\Delta P}{\Delta Pr} = \frac{P_f - P_i}{Pr_i - Pr_f} \quad (5)$$

In the case of bacteriocins, the yield of their production with regard to the biomass present was also calculated, which allowed the definition of specific productivity values of the biomass under different conditions:

$$Y_{BT/X} = \frac{\Delta BT}{\Delta X} = \frac{BT_f - BT_i}{X_f - X_i} \quad (6)$$

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 XP spreadsheet. Statistica 6.0 (StatSoft, Inc. 2001) and Simfit v.5.6.7 (kindly provided by Dr. W.G. Barsley of the Manchester University, UK and Dr. F.J. Burguillo of the Salamanca University, Spain) programs were used to evaluate the significance of the parameters estimated by the adjustment of the experimental values to the proposed mathematical models and the consistency of these equations.

RESULTS AND DISCUSSION

The preliminary assays of bacteriocin production on these residual medium (using similar bacteriocin producers), led to smaller pediocin production to those that were obtained in the commercial medium MRS [29]. These low levels of pediocin maybe due to the poor peptidic variety that present the peptones extracted without any specific proteolysis treatment. Therefore, in order to improve the yield of these protein sources, controlled enzymatic hydrolyses were carried out with proteases such as trypsin, pepsin and papain (data not shown).

L. lactis and P. acidilactici cultures in marine peptone media

In order to demonstrate the usefulness of the marine peptones obtained by means of enzymatic hydrolysis as a source of protein for the culture of micro-organisms and the production of bacteriocins, two LAB strains highly productive of nisin: *Lactococcus lactis* HD1 [19] and pediocin: *Pediococcus acidilactici* 1.02 [12, 30] were used.

The culture samples were taken at times corresponding to the exponential and asymptotic phases of the bacterial growth (10 and 24 hours for Lc HD1; 18 and 48 hours for Pc 1.02). Figure 1, the values of biomass (X) and bacteriocins (BT) obtained at the asymptotic phase are compared. In the case of *L. lactis*, the highest growths and nisin productions were obtained in the media where papain was used for hydrolysis of the protein material. In addition, this production was improved with the increase in the period of enzymatic action. Regarding the level of papain used, the Lc HD1 growth was higher with a low level of enzyme (1.25 mg enz/g prot) and the nisin production was indifferent to the enzyme concentration used. The other enzymes employed led to peptidic hydrolysates that promoted higher growths to those obtained on commercial medium, although pepsin hydrolysis yielded more efficient peptones for nisin production than the trypsin hydrolysis.

The results from Pc 1.02 growth and pediocin production (Figure 1, down) show the greater efficiency of the media with hydrolysed peptones, especially with papain and pepsin proteolysis.

Cultures on selected marine peptones

In the search to obtain a more detailed and formal description of the microbial growth in the marine peptone media, the nine cultures resulting from the combination of the two bacteria tested with the four best hydrolysates previously described and the initial medium without enzymatic hydrolysis (P0), were subsequently tested (Table 3). Also, MRS medium was used as a term of comparison. In these complete cultures the production of biomass, bacteriocins, lactic acid, acetic acid (data not shown), evolution of pH and the glucose and protein uptake were determined.

Figures 2 and 3 show the kinetics of Lc HD1 and Pc 1.02 cultures. These results are consistent with the assessment derived from the earlier screening. In the two bacteria tested, we can observe the increase of the growth and bacteriocin production on the media with a hydrolysed peptide source, in comparison with the unhydrolysed peptone and the commercial MRS medium. From a quantitative viewpoint, the numerical adjustment of the experimental data to the mathematical models proposed (Figures 2 and 3, continuous lines) generated the parametric estimations and the yields shown in Table 4. The equations proved to be consistent in all the cases (Fisher's F ; $\alpha=0.05$), and the parametric estimations passed Student's t -test ($\alpha=0.05$). On the other hand, all the values predicted by the non-linear adjustments produced high coefficients of linear correlation with the values really observed (Table 4).

Taking into account the numeric value of the parameters and the bacterial strains to study, we can indicate that:

1: The levels of maximum biomass (K), maximum growth rates (v_{mx}), maximum nisin production (BT_m) and maximum nisin rate production (v_{mb}) by *L. lactis*, favoured to the hydrolysed waste media (being the denominated medium PAP3 the most effective) with regard to the commercial broth (MRS) and to the waste media without hydrolysis (P0). Only in the lactic acid bioproduction the medium MRS was superior to the rest.

2: As in the previous case, the peptones sources obtained by enzymatic hydrolysis of wastewater from the industrial processing of octopus, especially PEP2, promoted the highest numeric values in K , v_{mx} , BT_m , v_{mb} , $Y_{X/Pr}$, $Y_{X/RS}$, $Y_{BT/Pr}$ and $Y_{BT/RS}$ of *Pediococcus*

acidilactici. However, the highest productive parameters of lactic acid (L_m , v_{ml} , $Y_{L/Pr}$ and $Y_{L/RS}$) corresponded to the complex protein sources formulated in the commercial broth.

CONCLUSIONS

The main conclusions generated by these preliminary results can be summarized in the two following points:

1: In coastal environments where industries dedicated to the production of marine foodstuffs are plentiful (octopus in our study), the large quantities of wastewater derived from their activity (which represent a serious environmental problem) provide, in a simple and rapid manner, sources of waste protein. These nutrients by means of enzymatic hydrolysis generate peptones which equal or surpass the production of biomass and bacteriocins on the high-priced medium recommended for the culture of lactic acid bacteria.

2: The highest production of biomass by *L. lactis* and nisin corresponded to the media which presented peptones hydrolysed with low concentration of papain and with short times of hydrolysis (4 hours). In the case of pediocin, the highest production corresponded to the media hydrolysed with papain, trypsin and pepsin and during not more than 10 hours. These results allow us to propose a simple and cheap treatment of valorisation for water with medium or high protein content in order to obtain peptones for micro-organism culture media.

ACKNOWLEDGEMENTS

Thanks to Ana Durán, Margarita Nogueira and Javier Fraguas for technical assistance. The English usage in the manuscript has been revised by J&J Barry Consulting, S.L. (Global Pharmaceutical Support, Vigo, Spain).

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Figure captions

Figure 1: Productions of biomass (X) and bacteriocins (BT) on waste water hydrolysates, by cultures of *L. lactis* (above) and *P. acidilactici* (below) at the asymptotic phase. Hydrolysis conditions (time and enzyme concentrations) are specified on the axes. Horizontal lines indicate the productions obtained on MRS medium.

Figure 2: Time-course of *L. lactis* cultures on media prepared with marine peptones as a peptide source (PAP 1: ○, PAP 2: □, PAP 3: ◇, PAP 4: ▽, P0: ■. Table 3), and on commercial MRS medium (●). The continuous lines represent the fits from the experimental data (points) to the mathematical models described in the text and the discontinuous lines represent the profiles without adjustment. X: biomass, BT: bacteriocin (nisin), RS: reducing sugars, Lact: lactic acid, Prot: proteins.

Figure 3: Time-course of *P. acidilactici* cultures on media prepared with marine peptones as a peptide source (PAP 1: ○, PEP 1: □, PEP 2: ◇, TRIP 1: ▽, P0: ■. Table 3), and on commercial MRS medium (●). The continuous lines represent the fits from the experimental data (points) to the mathematical models described in the text and the discontinuous lines represent the profiles without adjustment. BT: bacteriocin (pediocin). The rest of the keys as in fig. 2.

Figure 1

Figure 2

Figure 3

Tables

TABLE 1: Composition of culture media used in microbiological assays (g.l⁻¹).

	MP media ¹	MRS medium
Glucose	20.00	20.00
Yeast extract	4.00	4.00
Sodium acetate	5.00	5.00
Ammonium citrate	2.00	2.00
K ₂ HPO ₄	2.00	2.00
MgSO ₄	0.20	0.20
MnSO ₄	0.05	0.05
Tween 80	-	1.00
Meat extract	-	8.00
Bactopeptone	-	10.00
Marine peptone protein (Lowry)	10.00	-

(¹): Marine peptone from octopus defined in Text.

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

X :	Biomass. Dimensions: g.l^{-1}
t :	Time. Dimensions: h
K :	Maximum biomass. Dimensions: g.l^{-1}
X_0 :	Initial biomass. Dimensions: g.l^{-1}
v_{mx} :	Maximum growth rate. Dimensions: $\text{g.l}^{-1}.\text{h}^{-1}$
λ_x :	Growth lag phase. Dimensions: h
L :	Lactic acid. Dimensions: g.l^{-1}
L_m :	Maximum lactic acid. Dimensions: g.l^{-1}
v_{ml} :	Maximum lactic acid rate production. Dimensions: $\text{g.l}^{-1}.\text{h}^{-1}$
λ_l :	Lactic acid lag phase. Dimensions: h
BT :	Bacteriocin (pediocin or nisin). Dimensions: BU.ml^{-1}
BT_m :	Maximum bacteriocin. Dimensions: BU.ml^{-1}
v_{mb} :	Maximum bacteriocin rate production. Dimensions: $\text{BU.ml}^{-1}.\text{h}^{-1}$
λ_b :	Bacteriocin lag phase. Dimensions: h
P :	Product concentration (X , L , BT) formed by the microorganism. Dimensions: g.l^{-1} or BU.ml^{-1}
RS :	Reducing sugars concentration consumed by the microorganism. Dimensions: g.l^{-1}
Pr :	Protein concentration consumed by the microorganism. Dimensions: g.l^{-1}
$Y_{P/Pr}$:	Product formation / protein consumption. Dimensions: g product or BU / g protein
$Y_{P/RS}$:	Product formation / reducing sugars consumption. Dimensions: g product or BU / g reducing sugars
$Y_{BT/X}$:	Bacteriocin production / biomass production. Dimensions: BU / g biomass

TABLE 3: Selected media for kinetic studies of Lc HD1 and Pc 1.02 cultures

Enzyme	key	hydrolysis conditions for Lc HD1		hydrolysis conditions for Pc 1.02	
		time (h)	C (mg enz/g prot)	time (h)	C (mg enz/g prot)
Trypsin	TR 1	-	-	10	3.75
Pepsin	PEP 1	-	-	10	1.25
	PEP 2	-	-	4	3.75
Papain	PAP 1	4	1.25	10	12.50
	PAP 2	10	1.25	-	-
	PAP 3	24	1.25	-	-
	PAP 4	24	12.50	-	-
Without enzyme	P0	-	-	-	-

TABLE 4: Parametric estimations (see Table 3) corresponding to the kinetic models (1-6), applied to cultures of *L. lactis* and *P. acidilactici* on specified media (see Table 2). Intervals of correlation coefficients between observed and expected productions were: $r(X)=0.989-0.999$; $r(L)=0.983-1.000$; $r(BT)=0.991-1.000$.

STRAINS	MEDIA	BIOMASS (X)			LACTIC (L)			BACTERIOCIN (BT)			YIELDS (Y)						
		K	v_{mx}	λ_x	L_m	v_{ml}	λ_l	BT_m	v_{mb}	λ_b	$Y_{X/Pr}$	$Y_{X/RS}$	$Y_{L/Pr}$	$Y_{L/RS}$	$Y_{BT/Pr}$	$Y_{BT/RS}$	$Y_{BT/X}$
Lc HD1	PAP 1	2.559	0.521	4.851	4.654	0.582	3.775	53.729	7.889	4.588	1.331	0.282	2.578	0.546	28630	6067	21515
	PAP 2	2.495	0.554	5.088	4.716	0.710	4.354	49.031	11.408	5.154	1.133	0.278	2.289	0.561	22711	5563	20045
	PAP 3	2.583	0.501	5.078	4.504	0.799	4.940	63.503	12.055	5.716	0.996	0.325	1.744	0.569	23019	7506	23104
	PAP 4	2.270	0.419	4.808	4.948	0.829	4.746	58.120	16.549	5.750	1.260	0.285	2.739	0.619	31239	7061	24787
	MRS	1.171	0.211	3.951	5.836	0.828	4.033	31.961	5.474	4.959	1.071	0.125	5.528	0.645	27519	3209	25700
	P0	1.625	0.226	3.364	5.170	0.661	5.139	30.227	5.044	4.360	0.999	0.150	3.269	0.491	18600	2792	18612
Pc 1.02	PAP 1	3.114	0.172	5.849	6.920	0.264	6.200	271.19	21.230	7.531	2.179	0.230	4.723	0.499	178459	18839	81898
	PEP 1	3.434	0.232	6.141	6.659	0.333	3.781	294.19	28.725	8.839	2.746	0.272	5.244	0.519	226160	22394	82366
	PEP 2	3.511	0.287	7.431	6.642	0.302	2.945	307.04	27.521	8.352	4.301	0.242	8.683	0.488	364988	20527	84857
	TR 1	2.063	0.143	5.452	7.193	0.363	5.629	290.56	29.916	8.621	2.422	0.144	8.802	0.524	320837	19082	132463
	MRS	1.267	0.075	4.672	7.953	0.382	6.064	227.70	21.258	9.148	1.433	0.101	9.242	0.654	234967	16627	163972
	P0	2.438	0.131	7.039	6.435	0.299	6.254	137.79	7.519	8.077	2.803	0.201	7.584	0.543	153584	11006	54786