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Mathematical tools for objective comparison of microbial cultures Application to evaluation of 15 peptones for lactic acid bacteria productions

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Abstract

Using the logistic model as a starting point, a set of reparameterised equations were established which permit the easy calculation of the relevant parameters of microbial kinetics, together with their confidence limits, with the aim of establishing rigorous comparison between cultures under differing conditions. When the resource was used to evaluate the aptitude of peptones from diverse sources for the culture of lactic acid bacteria (LAB), a great variability was found, even among the results of commercial formulations with the same denomination. None of the peptones was able to maximise the growth – very active in fish peptones – and the production of the characteristic metabolites at the same time. Under these conditions, the application of the cluster analysis to kinetic parameters of proven descriptive capability becomes a useful exploratory method, which allows to decide combinations of protein sources apt to make compatible different potential purposes of LAB cultures. This way, it is possible to design factorial experiments to search optimum values for these purposes on the basis of hypothesis which avoid the selection of superfluous variables and inadequate domains.

Keywords: Logistic equation; Lactic acid bacteria; MRS media; Commercial peptones; Fish viscera peptones; Lactic acid; Nisin; Pediocin

1. Introduction

From the point of view of their industrial interest, lactic acid bacteria (LAB) are an important microbial group, due to their role in food fermentation and preservation, either as natural microbiota, or inocula added under controlled conditions. Among the bioactive molecules produced by LAB are lactic acid, acetic acid, ethanol, diacetyl, 2,3-butanediol and bacteriocins [1,2]. Bacteriocins are peptides with antimicrobial activity and have interest in alimentary industry as they are innocuous, sensitive to digestive proteases, and do not change the organoleptic properties of the food [3,4]. However, the large-scale production of LAB and bacteriocins is expensive due to the complex media – rich in protein hydrolysates – which they require for growth. Commercial media as MRS, TGE or APT solve the problem of protein sources, by means of products such as bactopeptone, tryptone, meat extract or yeast extract (sometimes all of them) in formulations which, however, reach high costs.

Even if these peptones are necessary for bacteriocin production [5–7], the efficiencies (substrate consumed/initial substrate) of these media are usually low, suggesting unbalanced proportions of nutrients [8]. Thus, the protein materials which remain in the media at the end of the incubation constitute superfluous expenditure and hinder the subsequent purification of the bacteriocins. The replacement of these proteins by inorganic sources of nitrogen does not produce acceptable results [9], nor is suitable the initially obvious solution of adjusting the initial protein level to the detected consumption [5]. It is this way because peptones do not represent simply a source of organic nitrogen, but rather a source of amino acids or peptides with specific roles, in such a way that only a fraction of the total added is really important [10–15]. So, the use of low-cost protein fractions will bring about a reduction in large-scale production costs. Furthermore, if food waste (as that generated by the processing of resources from marine origin) is used to obtain those protein fractions, a productive cycle is closed: recycling of a pollutant waste and obtaining products

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(bacteriocins) with high added value, useful for preservation of foodstuffs.

According to the definition of Green et al. [16], 'peptones' are water-soluble protein hydrolysates non-coagulable by heat. Commercial peptones used in microbiological media are mainly derived from casein, soy and meat. Peptones from marine origin are barely used today, in spite of their good results in some applications, as is the case with the production of proteases by *Bacillus subtilis* [17] or *Vibrio* species [18], with gastrine and epidermal growth factor (EGF) by mouse fibroblasts [19], glycerol by *Saccharomyces cerevisiae* [20], lactic acid bacteria [21–23], probiotic marine bacteria [24], bacteriocins [25,26] or microbial growth [27,28].

This study attempts to validate the use, in more general terms, of fish peptones, through an approximation which presents the following characteristics. (1) The study includes a comparison, from various angles, between the results obtained with 4 'marine' peptones specifically prepared for this purpose and 11 commercial formulations. (2) Microorganisms used were LAB, well known for a complex nutritional requirement which demands diversified peptide sources. (3) Comparisons were performed in the most rigorous way possible, using parametric estimations with biological significance and verified statistic reliability, obtained through the fitting of all the cases studied to the same mathematical models, whose pertinence was discussed at a formal level and verified through experimental results.

2. Materials and methods

2.1. Preparation of marine peptones from fish viscera

Raw materials used were viscera from swordfish (*Xiphias gladius*), shark (*Isurus oxyrhinchus*), thornback ray (*Raja clavata*) and yellowfin tuna (*Thunnus albacares*), sampled immediately after industrial processing and maintained at −20 °C until use. Storage did not exceed 15 days for all the materials. Visceral masses (stomach and intestine) were ground with equal weights of distilled water, and the homogenates, after steam flow stabilisation (101 °C/1 h), were treated in a centrifuge decanter at 7500 × *g*/15 min [25,26]. Supernatants, or marine peptones, were typified by determining the levels of total nitrogen, protein and total sugars, and stored at −20 °C until time of use for the formulation of culture media. Raw composition of these peptones is shown in Table 1.

Table 1
Main composition (g L^{-1}) of marine peptones (MP) from fish viscera

	Proteins (Lowry)	Total sugars	Total nitrogen
SF	18.7	0.8	3.8
S	31.3	2.2	10.7
TR	22.0	1.1	8.2
YT	23.6	1.5	4.5

SF: sword fish; S: shark; TR: thornback ray; YT: yellowfin tuna.

2.2. Microbiological methods

Microorganisms used as bacteriocin producers were *Pediococcus acidilactici* NRRL B-5627 (abbreviated key Pc 1.02), from Northern Regional Research Laboratory (Peoria, IL, USA), and *Lactococcus lactis* subsp. *lactis* (abbreviated key Lc HD1) isolated from salmon sausages and supplied by Dr. López Cabo (IIM-CSIC, Spain). *Carnobacterium piscicola* CECT 4020 (Spanish Type Culture Collection) and *Leuconostoc mesenteroides* subsp. *lysis* (kindly provided by Dr. Ray, University of Wyoming, Laramie, USA) were used as indicators in bacteriocin bioassays. Stock cultures were stored at −75 °C in MRS medium (Pronadisa, Hispanlab S.A., Spain) with 25% glycerol [8]. Inocula (1%, v/v) consisted of cellular suspensions from 24-h (Pc 1.02) and 12-h (Lc HD1) cultures on MRS medium, adjusted to an OD (λ = 700 nm) of 0.900.

The commercial protein sources here studied (Table 2) were always used at a concentration equivalent to that determined (Lowry) in MRS medium. In all cases, the initial pH was adjusted to 7.0 and the solutions sterilized at 121 °C, 15 min. Microorganisms were grown at 30 °C in 300 mL Erlenmeyer flasks with 200 mL of medium (optimal conditions for nisin and pediocin productions [29]), under orbital shaking at 200 rpm. Cultures were carried out in triplicate.

2.3. Analytical methods

At pre-established times, each culture was divided into two aliquots. The first one was centrifuged at 4000 × *g* for 15 min, the sediment washed twice and resuspended in distilled water to an appropriate dilution for OD measuring at 700 nm. Dry weight can then be estimated from a previous calibration curve. The corresponding supernatant was used for determination of proteins, lactic and acetic acids, and reducing sugars. The second aliquot was used for extraction and quantification of pediocin (produced by Pc 1.02) and nisin (produced by Lc HD1), using *C. piscicola* and *L. mesenteroides*, respectively, as indicators, according to previously described methods [30,31].

Other analytical determinations were—*total nitrogen*: method of Havilah et al. [32], applied to digests obtained through the classic Kjeldahl procedure. *Proteins*: method of Lowry et al. [33]. *Total sugars*: phenol–sulphuric reaction [34] according to the application of Strickland and Parsons [35] with glucose as a standard. *Reducing sugars*: 3,5-dinitrosalicylic method [36]. *Lactic and acetic acids*: HPLC, after membrane filtration of samples (0.22 μm Millex-GV, Millipore, USA), using an ION-300 column (Transgenomic, USA) with 6 mM sulphuric acid as a mobile phase (flow = 0.4 mL/min) at 65 °C and a refractive-index detector. All assays were carried out in duplicate.

2.4. Mathematical models

A widely accepted model for the macroscopic description of the microbial growth kinetics is the logistic equation [37–40], one advantage of which is the direct biological significance of its parameters. This model describes the biomass variation against time by means of the following differential equation, typical for