Valorisation of effluents obtained from chemical and enzymatic chitin production of *Illex argentinus* pen by-products as nutrient supplements for various bacterial fermentations

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ABSTRACT

The industrial production of chitin generates large volumes of protein effluents that need to be handled and depurated before discharge. The current study highlights the suitability of four effluents, derived from the chemical and enzymatic hydrolysis of *Illex argentinus* pen to produce β-chitin, as peptones for the growth and metabolite production of six bacteria with different nutrient requirements. Batch cultures were carried out determining the growths by dry weight and viable cell counts and modelling kinetics using the logistic equation. Two lactic acid bacteria were perfectly supported by alternative media formulated with chitin effluents and the results were better than those found in commercial ones. For the other four bacteria, the biomasses in chitin peptones were lower but the number of produced cells was similar to those defined using Marine medium (MM) and tryptone-soy broth (TSB). An economic assessment demonstrated the profitability achieved when commercial peptones are replaced by those generated from squid pen: reduction of costs by 6 times for lactic acid bacteria, 50-100 times for marine bacteria and 6-17 times for Gram (+) bacteria.

Keywords: microbial growth; waste-water treatment; bioconversion; peptones; *Illex argentinus* pen by-products; chitin production.

1. INTRODUCTION

The cephalopods global market has grown considerably in the last decades. The main fisheries targeting these species are located in the Southwest Atlantic region (Area 41, FAO) and Northwest Pacific region (Area 61, FAO) contributing to two-thirds of the total global catches [1]. Squids of the family Ommastephidae are distributed worldwide in the neritic and oceanic zone and constitute the most important group of cephalopods due to the volume of the fishing (80%) [2]. *Illex argentinus* and *Todarodes pacificus* are the main species and constitute 46% of the world catches. Spain is the second country exporting squid and cuttlefish and is also one of the largest consumers and importers of these species; in fact, during 2012 the Spanish fleet landed 22864 t [3]. *Illex argentinus* is a medium size species of squid (Castellanos, 1960) with a well-developed head surrounded by ten mobile appendices that is commonly a by-catch of the other trawl fishery for hake. Its skeleton is represented by a chitin structure called pen that is generally a by-product produced in industrial squid processing.

Chitin is, after cellulose, one of the most abundant polysaccharides in nature with support and protection functions in a variety of invertebrates (arthropods, mollusc, cnidarians, pogonophores) and also in the cellular wall of algae and fungi. Chitin occurs in crystalline state, X-ray spectra shows three polymorphic forms, α, β and γ. The squid pen is mainly composed of β-chitin in association with proteins. β-chitin is less abundant than α-chitin but could be obtained in an important amount from squid pens (31-49% on dry basis) [4]. There is little information on β-chitin, but it presents higher solubility in solvents and higher

reactivity regarding deacetylation and chemical modification than α-chitin [5,6]. In spite of being a renewable natural resource, the accumulation of squid pen is a source of contamination in coastal zones. The special characteristics of this biomaterial, such as its biodegradability and biocompatibility [7], make its valorisation an opportunity for industrial development in biotechnology and pharmaceutical processes and constitutes an effective solution for the environmental problem that it generates [8-10].

Nevertheless, the chemical or enzymatic processes for the isolation of chitin produce effluents with medium-large concentrations of proteins and high chemical oxygen demand (COD) which require specific handling for their depuration. To our knowledge, no reports on the valorisation of those effluents have yet been developed. A potential alternative could be their inclusion as ingredients (source of organic nitrogen) in the media for bacterial growth replacing commercial peptones. The fermentation media formulated to support microbial cultures contain various peptides, protein hydrolysates and amino acids that are generally denominated as peptones. In many bioprocesses, more than 50% of the production cost of fermentation products is due to the excessive market price of commercial nitrogen-rich supplements [11]. In this context, different marine and agrifood wastes have been successfully evaluated in recent years as crude nutrient supplements in various bioprocesses [12-15].

In the present manuscript, different protein effluents generated in the production of chitin from *I. argentinus* pens are used as alternative peptones for the growth of several bacteria. Thus, three well-known culture media (Man-Rogosa-

Sharpe: MRS, Marine medium: MM and tryptone-soy broth: TSB) are reformulated with the mentioned effluents in order to assess their capacity to support the production of lactic acid bacteria, marine probiotic bacteria and two common gram (+) bacteria.

2. MATERIALS AND METHODS

2.1. Production of chitin from I. argentinus pen by-products

Squid pen by-products from *Illex argentinus* were kindly provided (clean and frozen) by Cabomar S.A. (Marín, Spain) and stored at -4ºC until use. Prior to the subsequent application, the pens were washed, dried at 55ºC for 24 h, grinded and sieved with a mesh of 500 μm. Two types of processes were performed to isolate chitin from squid pens: alkaline treatment and enzymatic proteolysis (both in duplicate). In all cases, a glass-reactor of 5 L with pH and temperature control was used for pen deproteinisation with 400 g of initial substrate and 4 L of reagent (NaOH for alkaline and water plus enzyme for enzymatic hydrolysis). For the latter, three experiments were carried out with three different enzymes (from Novozymes, Nordisk, Bagsvaerd, Denmark): Alcalase 2.4L (2.4 Anson Unit/g, AU/g), Neutrase 0.8L (0.8 AU/g) and Esperase 8L (8 KNovo Protease Unit/g, KNPU/g). The concentration of enzyme was always 1% (v/w of pen) and the times of hydrolysis were 6 h. The rest of experimental conditions and the flow chart of pens processing are summarised in Figure 1. At the end of the proteolysis, solids (chitin) were separated of protein effluent (from here called as peptones) by filtration (20 μm) and stored at -20ºC up to media formulation. Thus, four types of peptones were obtained: 1) Peptone A from chemical processing, 2) Peptone B from alcalase hydrolysis,

3) Peptone C from esperase hydrolysis and 4) Peptone D from neutrase hydrolysis.

The basic composition of peptones is shown in Table 1. In addition, the amino acid content of these peptones were analysed according to the method of ninhydrin reaction using an amino acid analyzer according the method of Spackman et al. [16] (Table 2).

2.2. Enzyme proteolysis of pens

The hydrolysis degree (*H*, in %) was calculated according to the pH-Stat method defined by Adler-Nissen [17] applying the following equation:

$$H = \frac{B N_b}{\alpha M_p h_{tot}} \qquad (1)$$

where, B is the volume (mL) of 0.2 M NaOH consumed during hydrolysis; N_b is the normality of NaOH; M_p is the mass (g) of initial protein (N x 6.25); h_{tot} is the total number of peptide bonds available for proteolytic hydrolysis (8.6 meq/g), and α is the average degree of dissociation of the amino groups in the protein substrate and it was determined with:

$$\alpha = \frac{10^{pH-pK}}{1+10^{pH-pK}} \qquad (2)$$

The pK value is dependent on the temperature of hydrolysis (in K degrees) and can be estimated according to the expression:

$$pK = 2400\left(7.8 + \frac{298 - T}{298\,T}\right) \quad (3)$$

Finally, the kinetics data of the *I. argentinus* pen hydrolysis (*H*) were modelled by means of the Weibull equation [18]:

$$H = H_m\left\{1 - \exp\left[-\ln 2\left(\frac{t}{\tau}\right)^{\beta}\right]\right\} \quad \text{with} \quad v_m = \frac{H_m\,\beta\,\ln 2}{2\,\tau} \quad (4)$$

where, H is the degree of hydrolysis (%); t is the time of hydrolysis (min); H_m is the maximum degree of hydrolysis (%); β is a parameter related with the maximum slope of pen hydrolysis (dimensionless); τ is the time required to achieve the semi-maximum degree of hydrolysis (min) and v_m is the maximum hydrolysis rate at the τ-time (% min^{-1}).

2.3. Microbiological methods and culture media

The bacteria used in the present work, acronyms and their corresponding commercial media were: *Lactobacillus casei ssp. casei* CECT 4043 (Spanish Type Culture Collection), Lb 1, MRS; *Lactobacillus plantarum* CECT 220 (Spanish Type Culture Collection), Lb 2, MRS; *Pseudomonas fluorescens* DIFR AH-2 (Danish Institute for Fisheries Research), Pf, MM; *Phaeobacter* sp. DIFR 27-4 (Danish Institute for Fisheries Research), Ph, MM; *Bacillus subtilis* CECT 35 (Spanish Type Culture Collection), Bs, TSB; *Staphylococcus epidermidis* CECT 231 (Spanish Type Culture Collection), Se, TSB.

Stock cultures were stored at –80ºC in the adequate commercial media for each case (MRS: Pronadisa, Hispanlab S.A., Spain; MM: Difco, Becton, Dickinson and Company, MD, USA; TSB: Panreac Química, Spain) with 25% glycerol. Inocula (0.5% w/v) consisted of cellular suspensions from 12-16 h cultures on control medium. Tables A, B and C (supplementary material) summarise the composition of the culture media. Yeast extract was always provided by Cultimed (Panreac Química, Spain). The protein concentrations in the media formulated with peptones from *I. argentinus* pen by-products were established by replacing the Lowry protein level in the peptones present in MRS (10 g/L), MM (2.6 g/L) and TSB (11 g/L). Fermentations were carried out in duplicate using 300 mL Erlenmeyer flasks with 150 mL of medium at 30ºC (Lb 1, Lb 2 and Bs), 22ºC (Pf and Ph) or 37ºC (Se) and 200 rpm of orbital shaking. In all cases, the initial pH was adjusted to 6.0, 7.5 and 7.3 with NaOH 5 N for MRS, MM and TSB alternative media. Culture broths media were finally sterilised separately at 121ºC for 15 min.

2.4. Analytical methods

At pre-determined times, samples from each culture were divided into two aliquots. The first aliquot was used for quantifying viable cells by means of a plate count technique in MRS, MM or TSB agar medium depending on the bacterial strain. Serial tenfold dilutions were prepared in peptone-buffered solutions, and 0.1 mL samples were plated in triplicate, incubated at the required temperature (22, 30 or 37ºC) for 48-72 h, and manually counted. For clarity, the results were expressed as $G=\ln(N/N_0)$, where N is the colony-forming

units per mL (cfu/mL) and N_0 is the initial colony-forming units per mL (cfu/mL). The second aliquot was centrifuged at 3,273 × *g* for 15 minutes, from which the supernatant was used for determining, in all cases, the proportions of proteins. In TSB cultivations reducing sugars were also quantified and, for MRS fermentations, lactic and acetic acid as well as reducing sugars were additionally measured. The corresponding sediment was washed twice and resuspended in distilled water at an appropriate dilution to measure the optical density at 700 nm (in arbitrary units, AU).

Reducing sugars were quantified by means of a 3,5-dinitrosalicylic reaction [19]. Soluble proteins were obtained using the method of Lowry et al. [20]. Total nitrogen was determined by the method of Havilah et al. [21], applied to peptone digests obtained by the classic Kjeldahl procedure. Total sugars content in peptones were analysed by the method of Dubois et al. [22] according to the application of Strickland and Parsons [23]. Lactic and acetic acids were measured by HPLC, after the samples had been membrane filtered (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 65ºC and a refractive-index detector. All the analysis determinations were done in duplicate.

2.5. Mathematical modelling of cultivations

The non-linear trends obtained for bacteria biomass production (*X*), cell formation (*G*), lactic acid production (*L*) and acetic acid production (*A*) were fitted to the logistic equation [24]:

$$P = \frac{P_m}{1 + \exp\left[2 + \frac{4v_P}{P_m}(\lambda_P - t)\right]} \quad (5)$$

where, P is the product determined (X, G, L or A); P_m is the maximum product production (g/L for X, L and A and dimensionless for G); v_P is the maximum production rate (g L^{-1} h^{-1} for X, L and A and h^{-1} for G); λ_P is the products lag phase (h). In addition, the yields of productions on reducing sugars (Y_P/Y_{Rs}) and proteins uptakes (Y_P/Y_{Pr}) were also calculated.

2.6. Numerical and statistical analyses

Fitting procedures and parametric estimations calculated from the results were carried out by minimising the sum of quadratic differences between the observed and model-predicted values, using the non-linear least-squares (quasi-Newton) method provided by the macro-'Solver' of the Microsoft Excel spreadsheet. Confidence intervals from the parametric estimates (Student's t test) and consistence of mathematical models (Fisher's F test) were evaluated by "SolverAid" macro (Levie's Excellaneous website:http://www.bowdoin.edu/~rdelevie/excellaneous). A one-way analysis of variance (ANOVA) with the Tukey post hoc test (P=0.05) was used to determine whether significant differences were observed between parameters and data comparisons.

3. RESULTS AND DISCUSSION

3.1. Effluents production and composition

The optimal experimental conditions used in this work were obtained in a set of factorial designs previously performed to optimise the production of chitin from *I. argentinus* pen (data unpublished). The theoretical kinetics of protein hydrolysis mediated by proteases, under the conditions shown in Figure 1, are displayed in Figure 2. The values of parameters from equation (4) indicated that esperase led to the highest (H_m=22.30±0.07%) and fastest (v_m=0.124±0.001% min^{-1}) hydrolysis in comparison to alcalase (H_m=20.59±0.39% and v_m=0.088±0.001% min^{-1}) and neutrase (H_m=0.72±0.01% and v_m=0.016±0.001% min^{-1}) processing ($p<0.05$ from ANOVA). The hydrolysis degree of alkaline treatment was not determined by the pH-stat method because, in this case, the effect of NaOH on pen did not modify significantly the pH values over time.

Chemical composition of chitin effluents is shown in Table 1 and amino acid presence in chitin eflluents is summarised in Table 2. Esperase and NaOH application to squid pen yielded the major release of protein material into the filtrate-liquid fraction (58-59 g/L) followed by alcalase (51 g/L) and neutrase (24 g/L). A similar sequence was observed in terms of total nitrogen concentration. As expected this response was in agreement with the results of hydrolysis kinetics previously reported. No remarkable values of total and reducing sugars (less than 1 g/L) were detected in peptones (Table 1). Regarding amino acids, some differences in composition were found in the squid peptones. Neutrase treatment of pens released higher percentage of Lys, Leu and Glu as well as lower levels of Asp, Nleu and Gly than the other enzymes applied, while alcalase and esperase hydrolysis yielded a similar proportion of amino acids ($p<0.05$). The differences between enzyme treatments are in concordance with

the differences observed between the H_m values and the soluble protein extracted in the liquid phase. These values of maximum hydrolysis were similar for alcalase and esperase and much greater than for neutrase ($p<0.05$).

The commercial peptones showed greatly varied amino acid patterns (Table 2). Higher percentages of Gly and Glu and lower of Asp and Tyr were observed in Soy peptone, Bactopeptone and Meat extract ($p<0.05$). In Tryptone, proportions of Glu and Gly were very high and very low, respectively, in comparison with the other ones ($p<0.05$). Presence of OHlys and OHPro was significant in Meat extract and non-existent in most others. In general, the peptones developed here presented a good balance in the amino acid content even those that are essential in many bacteria (*L. plantarum*, *L. casei*, etc.) as Ile, Leu, Cys, Glu or Val. However, the essential amino acids requirements vary widely among bacteria and also between strains. No data about the essential amino acids needed for the strains used in the present work have been reported.

3.2. Growth of lactic acid bacteria on peptones from pens

L. plantarum (Lb 2) and *L. casei* (Lb 1) were selected for this approach since they have proved to have excellent technological properties including probiotic characteristics [25,26] and fermentation starters ability [11,27,28]. As an example, the complete results of Lb 2 in alternative media and MRS are represented in Figure 3. Experimental data of bioproductions (biomass as dry weight and cell formation jointly with lactic and acetic acid) were accurately modelled by equation (5). All parametric determinations for Lb 1 and Lb 2 are shown in Tables 3 and 4. For both strains, tested media and quantified

variables, the description of fermentations time-course by the logistic equation was almost perfect for all kinetic phases (R^2 variation from 0.971 to 0.999). The consistency of fits was also confirmed ($p<0.005$ from F-Fisher test). Furthermore, all parameters were statistically significant (t-Student test).

The maximum productions (X_m, G_m, L_m and A_m) were generally higher in the alternative media but the differences between them and the control medium (MRS) were not statistically significant ($p>0.05$). Only in the case of X_m for Lb 2 the presence of pen peptones significantly improved the maximum biomass production ($p<0.05$). On the contrary, the rate parameter (v_p) for all bioproductions and for the two lactic acid bacteria were numerically greater in MRS but without significant differences between culture media ($p>0.05$). A similar lack of significance was also observed in the comparison between cultures for the lag phases (λ_p). The experimental pH-trends and reducing sugars uptakes for each media and bacteria were indistinguishable. The best productivities (based on $Y_{P/Rs}$ and $Y_{P/Pr}$ yields) were found mainly in the media based on enzymatic hydrolysis of squid pen (especially medium C).

Lactic acid bacteria is perhaps the most adequate microorganism for the evaluation of food wastes as microbial nutrients due to they are fastidious bacteria that demand a semi-synthetic complex growth media [29,30] including a varied source of organic nitrogen [31,32] which cannot be replaced by an inorganic one [33]. The importance of the origin of the peptones for these bacteria has been also validated in the past [34,35]. Peptones obtained by enzymatic hydrolysis of wastewater from the industrial processing of octopus

showed their effectiveness in promoting the production of pediocin and nisin by two lactic acid bacteria (*Pediococcus acidilactici* and *Lactococcus lactis*) [36]. A previous study comparing several peptones from different origins, denominations and commercial brands led to a wide range of productive yields for the formation of bacteriocins and the growth of lactic acid bacteria [35]. In this context, protein substrates extracted from tuna heads and cod or tilapia viscera with varying degrees of hydrolysis have been reported as fundamental ingredients for complex media [14,37,38].

Our results have shown the validity of chitin production effluents to substitute commercial peptones in the formulation of MRS media for the growth of two lactic acid bacteria as *L. casei* and *L. plantarum*. As has been previously indicated by different authors [14,39], peptones obtained from food wastes or effluents from bioprocesses could be used to produce lactic acid bacteria particularly suitable for the development of halal and kosher products.

3.3. Production of marine probiotic on peptones from pens

Marine medium is a formulation designed for the specific growth of bacteria isolated from a marine environment. It is not a very nutritive broth, only 6 g/L of protein substrate (yeast extract and peptone), several mineral salts to simulate sea water but without source of sugars. Our formulation is the simplest and most cost-effective that can be defined: filtrate sea water plus 1 g/L of yeast extract and 2.6 g/L of Lowry-based protein from the effluents from squid pen proteolysis. In this context, Ph and Pf are two marine bacteria that have reported probiotic features in aquaculture [40-42]. This is the main reason for

their inclusion in the current study. They have potential applicability to the large scale and therefore low-cost media are required to achieve massive growths of such bacteria.

Figure 4 shows the experimental results for the selected case of Pf and fitting profiles of X and G variables to logistic equation (5). As in the previous section, the predictive ability of equation (5) to describe the experimental data of Ph and Pf was very high with determination coefficients always greater than 0.964 (Table 5). Total significance of kinetic parameters was also found. In both bacteria the biomass growth was significantly maxima in MM ($p<0.05$) and lower in the other media without significant differences between residual peptones from alcalase and esperase hydrolysis ($p>0.05$). Effluents from alkaline and neutrase treatments were the least effective for Pf and Ph, respectively. Although the capacity of the new media was too inferior to promote biomass production, it was quite remarkable and comparable to those obtained in formulations containing peptones from fish wastes [43].

However, the production of viable cells was equal in all media and significant differences were not observed ($p>0.05$). Similar findings were defined for the rest of the numerical parameters from biomass and cell growth determination. The differences between the results of X_m and G_m in the different media (Table 5) may be due to the fact that cells obtained in the alternative media are heavier than those produced in the control medium. This behaviour is in concordance with the differences reported in the growth of *Vibrio anguillarum* and *L. plantarum* using several peptones extracted from fish viscera wastes [13,44]. In

relation to the production yields we can indicate that media C and A showed the best productivities for Pf and MM and medium A for Ph.

3.4. Growth of B. subtilis and S. epidermidis on peptones from pens

TSB is a generic bacterial culture media that is extensively utilised in all microbiological labs around the world. It is formulated with a high relation C/N and high peptone content: 17 g/L of casein hydrolysate and 3 g/L of vegetal peptone. This level of protein material is sufficiently attractive to select such a nutritive broth in order to assess the substitutive validity of our peptones. Two common Gram (+) bacteria, Se and Bs, were also selected to be grown in TSB and low-cost media. The cultivation kinetics of Se (as example of TSB evaluations) is shown in Figure 5. The time-course of pH and glucose consumption in media A-D manifested similar trends to those defined by TSB.

The greatest values of X_m and G_m by Bs and Se were achieved in TSB and the peptones based on enzyme application were superior to those generated by chemical deproteinisation (Table 6). The values of λ_X and λ_G were not statistically significant in all kinetics because growth latencies were not observed at the beginning of bacterial growth. The yields in both bacteria for biomass production (as dry weight) were similar to the results of X_m. The values of $Y_{G/Rs}$ and $Y_{G/Pr}$ were less classifiable.

Different peptones derived from food marine wastes and agro-residues have been studied to cultivate *B. subtilis* strains in cost-effective media. Ellouz et al. [45] using protein substrates from fish achieved good results in applications

such as the production of proteases. Other enzymes (xylanase and keratinase) were produced using solid state fermentation in biowastes such as bran and straw fractions from wheat and maize as well as horn meal [46,47]. The growth of Bs was also perfectly maintained by peptones from waste chicken feathers [48] and cowtail ray viscera [49]. Recently, the production of a biosurfactant in soybean co-products has been reported [50].

In the case of Se, only three references to the use of wastes as ingredients for culture media have been published in literature. Qureshi and Dahot [51] described the use of molasses as carbon source for proteases production by *S. epidermidis* EFRL 12. Tuna processing wastes were evaluated for lipase production by *S. epidermidis* isolated from shrimp gut. Finally, fish protein hydrolysates from hake filleting wastes yielded excellent properties for the growth of four bacteria including *B. subtilis* and *S. epidermidis* [52].

3.5. Preliminary economical evaluation of effluents from chitin production

Based on the prices of commercial peptones, ingredients and culture media, we have calculated a reduction in the cost that the use of alternative protein source obtained from chitin production process supposes. In this context, the values of X_m from Tables 3 to 6 together with the volumes of culture were employed to calculate the bacterial biomass (in grams) produced in each fermentation. Taking into account the cost (in €) of the ingredients used for each cultivation, it is simple to join both variables and to establish the ratio €/g.

We have assumed that the production of such peptones has no cost due to the fact that they are generated as a wastewater from the industrial process to obtain valuable biopolymers as chitin and chitosan. The producers of chitin/chitosan must handle such effluents by means of a waste manager (with a high cost for the producers) or by applying solutions for its depuration/reuse/valorisation. In both cases, our valorising strategy is attractive in order to reduce the cost of handling by producing high value-added products such as lactic acid bacteria or marine probiotic bacteria.

The cost of lactic acid bacteria decreased almost six times when peptones A, B, C and D were presented in the MRS medium in substitution for commercial bactopeptone and beef extract. The case of marine probiotics bacteria was more spectacular because the cost reduction was 70 times for Ph in all media, 50 times for Pf in medium A and 100 times for Pf in medium D. Finally, commercial TSB was also economically improved replacing casitone and soy peptone by alternative peptones: from 6 to 16 times (peptone A to peptone D) for Bs and from 11 to 17 times (peptone A to peptone D) for Se. Similar results were obtained when the costs of production were calculated per maximum viable cell formation (parameter-G_m) (data not shown). In a previous report, the production of hyaluronic acid by *Streptococcus zooepidemicus* was halved including peptones from fish viscera by-products instead of tryptone [11]. No more comparisons with literature data could be made due to this kind of economical evaluations being unexplored.

4. CONCLUSIONS

An interesting alternative to the valorisation of effluents produced from chitin extraction of *I. argentinus* pens has been developed. Inexpensive peptones obtained by alkaline and enzymatic hydrolysis of the pens were used as an effective nitrogen source for the cultivation of bacteria from different ecological niches in substitution for the commercial ones. In all cases, the reduction of costs for the production of bacteria using alternative peptones was remarkable (*e.g.*, up to 100 times for marine probiotic bacteria). It was demonstrated that the effluents from chitin production could find widespread application in microbiological practice as a low-cost source of peptones in bacterial growth media.

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FIGURE CAPTION

Figure 1: Scheme of chitin and peptone production from *I. argentinus* pen by-products using chemical and enzymatic deproteinisation processing.

Figure 2: Theoretical kinetics of *I. argentinus* pen hydrolysis obtained by modelling the experimental data (not shown) with the Weibull equation (4) for the three enzymes applied under the conditions described in Figure 1.

Figure 3: Cultures of Lb 2 grown on different media formulated with peptones obtained from effluents of the chitin production from *I. argentinus* pen by-products. MRS medium was used as control. ○: peptone A, □: peptone B, △: peptone C, ▽: peptone D and ●: MRS. Experimental data of biomass (X), lactic acid (L), acetic acid (A) and cellular growth (G) were fitted to the equation (5). Reducing sugars (Rs) and proteins (Pr) uptakes as well as pH trends were also shown. The confidence intervals of experimental data (for two replicates) were in all cases less than 10% of the experimental mean value and omitted for clarity.

Figure 4: Cultures of Pf grown on different media formulated with peptones obtained from effluents of the chitin production from *I. argentinus* pen by-products. Marine medium (MM) was used as control. ○: peptone A, □: peptone B, △: peptone C, ▽: peptone D and ●: MM. Experimental data of biomass (X) and cellular growth (G) were fitted to the equation (5). Proteins (Pr) uptakes and pH trends were also shown. The confidence intervals of experimental data (for two replicates) were in all cases less than 10% of the experimental mean value and omitted for clarity.

Figure 5: Cultures of Se grown on different media formulated with peptones obtained from effluents of the chitin production from *I. argentinus* pen by-products. TSB was used as control. ○: peptone A, □: peptone B, △: peptone C, ▽: peptone D and ●: TSB. Experimental data of biomass (X) and cellular growth (G) were fitted to the equation (5). Reducing sugars (Rs) and proteins (Pr) uptakes as well as pH trends were also shown. The confidence intervals of experimental data (for two replicates) were in all cases less than 10% of the experimental mean value and omitted for clarity.

TABLE CAPTIONS

Table 1. Main composition (g/L) of the effluents obtained from the chitin production using *I. argentinus* pen as substrate. The error associated is the confidence intervals for n=2 and α=0.05.

Table 2. Percentage of amino acids (AA) contents on organic nitrogen sources (peptones) for the cultivation of the bacteria proposed in the current study. The error associated is the confidence intervals for n=2 and α=0.05.

Table 3. Parametric estimations corresponding to the equation (5) utilised to model the production of biomass, cells formation, lactic and acetic acids by Lb 1 in the media described in Table A (supplementary material). Error values are the confidence intervals (α=0.05). R^2= determination coefficients between experimental and predicted data. Production yields are also summarized.

Table 4. Parametric estimations corresponding to the equation (5) utilised to model the production of biomass, cells formation, lactic and acetic acids by Lb 2 in the media described in Table A (supplementary material). Error values are the confidence intervals (α=0.05). R^2= determination coefficients between experimental and predicted data. Production yields are also summarized.

Table 5. Parametric estimations corresponding to the equation (5) utilised to model the production of biomass and cells formation by Pf and Ph in the media described in Table B (supplementary material). Error values are the confidence intervals (α=0.05). R^2= determination coefficients between experimental and predicted data. Production yields are also summarized.

Table 6. Parametric estimations corresponding to the equation (5) utilised to model the production of biomass and cells formation by Bs and Se in the media described in Table C (supplementary material). Error values are the confidence intervals (α=0.05). R^2= determination coefficients between experimental and predicted data. NS: not significant. Production yields are also summarized.

Table 1

	Peptone A	Peptone B	Peptone C	Peptone D
Soluble protein	58.7±0.51	51.3±1.69	58.3±0.39	24.4±0.70
Reducing sugars	0.46±0.02	0.36±0.03	0.37±0.04	0.24±0.03
Total sugars	0.73±0.07	0.57±0.00	0.57±0.01	0.41±0.01
Total nitrogen	9.31±0.22	8.16±0.20	10.38±0.31	3.78±0.10

Table 2

AA	Peptone A	Peptone B	Peptone C	Peptone D	Soy Peptone	Bactopeptone	Meat extract	Tryptone
Asp	9.71±0.06	8.73±0.19	9.08±0.12	7.62±0.27	6.92±0.64	5.22±0.06	6.20±0.19	6.40±0.04
Thr	3.37±0.34	4.09±0.36	4.11±0.29	3.78±0.24	2.83±0.04	1.93±0.12	3.12±0.40	3.99±0.19
Ser	5.19±0.25	5.45±0.06	5.42±0.24	5.32±0.36	4.86±0.23	4.05±0.20	5.01±0.25	6.02±0.18
Glu	5.46±0.28	5.45±0.09	5.03±0.31	6.55±0.02	11.91±0.16	8.47±0.65	10.96±0.11	16.63±0.33
Gly	15.10±1.25	12.49±0.58	13.45±0.19	11.72±0.43	26.82±0.81	36.63±0.83	18.42±0.78	3.28±0.13
Ala	15.63±0.79	14.11±0.70	14.57±0.28	14.29±0.49	10.67±0.22	11.38±0.49	7.97±0.32	4.35±0.10
Cys	0.61±0.06	1.58±0.34	1.13±0.20	0.98±0.43	0.42±0.17	0.23±0.07	0.50±0.11	0.67±0.16
Val	5.32±0.26	5.18±0.63	5.04±0.44	5.56±0.72	3.46±0.01	2.40±0.00	4.00±0.16	6.09±0.05
Met	1.51±0.19	1.41±0.08	1.61±0.05	1.26±0.15	1.03±0.09	0.94±0.22	1.45±0.01	2.21±0.11
Ile	1.92±0.08	1.76±0.23	1.83±0.12	2.32±0.24	2.17±0.06	1.21±0.07	2.13±0.41	3.68±0.12
Leu	5.50±0.61	6.20±0.41	6.16±0.31	8.17±0.28	4.32±0.07	2.75±0.02	5.18±0.44	7.72±0.05
Nleu	3.19±0.99	3.07±0.08	2.79±0.63	0.60±0.15	1.82±0.01	1.29±0.10	6.63±0.34	8.44±0.32
Tyr	5.40±0.94	6.41±0.28	6.36±0.42	5.41±0.17	0.75±0.13	0.43±0.02	1.62±0.06	1.16±0.21
Phe	1.72±0.05	2.61±1.01	2.30±0.93	2.93±0.43	2.46±0.26	1.77±0.09	2.81±0.22	3.60±0.67
OHlys	-	-	-	-	-	-	0.44±0.02	-
His	5.88±0.45	6.33±0.33	6.33±0.31	6.67±0.19	1.07±0.13	0.79±0.26	1.18±0.07	1.74±0.21
Lys	2.29±0.12	3.35±0.11	3.08±0.03	4.23±0.13	4.81±0.24	4.05±0.12	3.98±0.34	5.96±0.12
Arg	1.29±0.22	1.58±0.06	1.49±0.21	1.43±0.04	4.27±0.04	4.79±0.16	3.67±0.03	2.30±0.20
OHPro	-	-	-	-	-	-	4.81±0.30	6.19±0.13
Pro	10.90±0.28	10.20±0.41	10.23±0.30	11.16±0.15	9.40±0.57	11.67±1.07	9.92±0.98	9.57±0.61

Nleu: norleucine; **OHlys:** hydroxylysin; **OHPro:** hydroxyproline.

Table 3

	Lb 1				
Parameters	Medium A	Medium B	Medium C	Medium D	MRS
X_m	4.61±0.44	4.80±0.37	4.50±0.35	4.62±0.37	4.27±0.28
v_x	0.383±0.119	0.411±0.115	0.396±0.113	0.389±0.109	0.603±0.217
λ_x	6.47±1.96	5.75±1.70	5.84±1.70	5.83±1.74	7.00±1.39
R^2	0.989	0.992	0.992	0.992	0.992
$Y_{X/Rs}$	0.200	0.226	0.215	0.212	0.195
$Y_{X/Pr}$	3.05	3.23	3.53	3.46	2.72
G_m	11.08±0.20	11.29±0.28	11.39±0.31	10.77±0.63	11.37±0.26
v_G	1.65±0.18	1.48±0.20	1.95±0.35	2.25±0.87	2.11±0.31
λ_G	4.12±0.41	3.73±0.56	4.01±0.59	5.00±1.08	4.80±0.46
R^2	0.999	0.999	0.998	0.990	0.999
$Y_{G/Rs}$	0.479	0.504	0.495	0.467	0.534
$Y_{G/Pr}$	7.29	7.21	8.13	7.64	7.45
L_m	13.18±1.28	14.62±1.68	13.68±1.78	14.93±2.01	14.37±0.96
v_L	1.15±0.40	1.13±0.41	1.11±0.49	0.972±0.323	1.17±0.25
λ_L	6.09±2.10	5.59±2.44	5.60±2.82	5.35±2.63	6.69±1.35
R^2	0.999	0.999	0.998	0.990	0.999
$Y_{L/Rs}$	0.621	0.659	0.656	0.655	0.681
$Y_{L/Pr}$	9.45	9.42	10.78	10.71	9.50
A_m	2.35±0.44	2.89±0.63	2.88±0.74	3.17±1.10	2.71±0.52
v_A	0.135±0.046	0.141±0.039	0.135±0.033	0.139±0.044	0.139±0.035
λ_A	6.19±3.05	6.74±2.85	8.29±2.66	6.95±3.67	7.58±2.48
R^2	0.980	0.983	0.986	0.971	0.985
$Y_{A/Rs}$	0.107	0.120	0.117	0.126	0.119
$Y_{A/Pr}$	1.63	1.71	1.92	2.06	1.66

Table 4

	Lb 2				
Parameters	Medium A	Medium B	Medium C	Medium D	MRS
X_m	2.90±0.09	2.62±0.15	2.50±0.13	2.71±0.24	2.31±0.16
v_x	0.282±0.036	0.228±0.050	0.221±0.043	0.245±0.085	0.256±0.083
λ_x	6.05±0.69	5.35±1.32	5.46±1.16	5.12±2.07	5.99±1.56
R^2	0.999	0.995	0.996	0.993	0.991
$Y_{X/Rs}$	0.132	0.125	0.121	0.127	0.101
$Y_{X/Pr}$	1.59	1.66	1.84	1.84	1.24
G_m	11.85±0.80	12.18±0.56	12.11±0.40	11.94±0.89	11.34±0.44
v_G	1.25±0.40	1.70±0.45	1.40±0.23	1.71±0.74	1.75±0.41
λ_G	3.83±1.66	3.71±1.07	3.99±0.80	4.12±1.71	3.93±0.86
R^2	0.991	0.994	0.998	0.985	0.996
$Y_{G/Rs}$	0.557	0.576	0.581	0.547	0.514
$Y_{G/Pr}$	6.74	7.66	8.82	7.88	6.32
L_m	16.08±2.08	15.50±1.94	15.58±1.86	15.72±1.58	15.85±1.34
v_L	0.993±0.276	0.997±0.299	0.968±0.257	1.11±0.31	1.19±0.29
λ_L	6.04±2.32	5.46±2.40	5.70±2.19	5.34±2.07	6.27±1.70
R^2	0.989	0.988	0.990	0.990	0.993
$Y_{L/Rs}$	0.742	0.749	0.752	0.727	0.743
$Y_{L/Pr}$	8.98	9.96	11.42	10.48	9.13
A_m	1.76±0.70	1.17±0.34	1.14±0.74	1.03±0.18	1.03±0.25
v_A	0.075±0.013	0.063±0.015	0.085±0.033	0.051±0.015	0.050±0.018
λ_A	14.05±2.43	12.68±2.29	13.67±2.66	3.31±3.04	4.98±3.67
R^2	0.994	0.986	0.999	0.980	0.973
$Y_{A/Rs}$	0.053	0.045	0.051	0.043	0.047
$Y_{A/Pr}$	0.647	0.604	0.780	0.616	0.578

Table 5

	Pf				
Parameters	Medium A	Medium B	Medium C	Medium D	MM
X_m	0.396±0.023	0.487±0.011	0.486±0.011	0.521±0.038	0.711±0.044
v_X	0.035±0.007	0.047±0.004	0.042±0.003	0.040±0.007	0.058±0.012
λ_X	8.32±1.13	7.36±0.48	8.51±0.43	9.42±1.23	5.61±1.35
R^2	0.997	0.999	0.999	0.996	0.995
$Y_{X/Pr}$	1.84	1.30	2.41	0.93	1.37
G_m	14.71±0.91	14.93±1.68	14.75±0.77	14.70±0.95	14.99±0.81
v_G	1.34±0.31	1.29±0.55	1.38±0.29	1.27±0.31	1.31±0.28
λ_G	6.27±1.33	4.77±2.61	5.98±1.15	5.10±1.48	4.33±1.28
R^2	0.994	0.980	0.996	0.994	0.995
$Y_{G/Pr}$	66.20	38.82	73.36	25.87	28.70
	Ph				
X_m	0.733±0.078	0.829±0.040	0.760±0.037	0.677±0.023	1.14±0.09
v_X	0.100±0.057	0.110±0.057	0.101±0.026	0.069±0.010	0.107±0.033
λ_X	7.14±2.28	6.85±1.06	6.72±1.05	6.73±0.74	6.69±1.74
R^2	0.979	0.996	0.996	0.998	0.992
$Y_{X/Pr}$	0.990	0.384	0.332	0.426	1.10
G_m	9.21±0.66	9.61±1.13	10.07±0.73	10.23±1.34	10.42±1.37
v_G	1.55±0.73	1.23±0.77	1.16±0.42	1.31±0.89	1.14±0.71
λ_G	6.07±1.55	4.38±2.75	4.22±1.75	6.09±2.92	4.97±3.11
R^2	0.988	0.965	0.987	0.968	0.964
$Y_{G/Pr}$	13.97	4.30	4.21	6.46	9.46

Table 6

	Bs				
Parameters	Medium A	Medium B	Medium C	Medium D	TSB
X_m	0.395±0.064	0.846±0.129	0.841±0.109	1.08±0.15	1.47±0.09
v_X	0.064 (NS)	0.060±0.034	0.069±0.038	0.061±0.022	0.085±0.033
λ_X	2.28 (NS)	0.552 (NS)	0.873 (NS)	1.33 (NS)	0.385 (NS)
R^2	0.907	0.953	0.960	0.977	0.940
$Y_{X/Rs}$	0.122	0.349	0.313	0.387	0.551
$Y_{X/Pr}$	0.399	0.595	0.592	0.829	0.895
G_m	6.12±1.11	7.64±1.11	7.91±1.00	7.34±1.08	8.08±0.67
v_G	0.687±0.665	0.605±0.351	0.550±0.249	0.706±0.489	0.781±0.305
λ_G	1.31 (NS)	1.43 (NS)	0.892 (NS)	1.47 (NS)	1.44 (NS)
R^2	0.902	0.956	0.969	0.942	0.981
$Y_{G/Rs}$	2.67	2.91	2.88	3.05	2.99
$Y_{G/Pr}$	8.71	4.96	5.44	6.53	4.86
	Se				
X_m	1.27±0.07	1.89±0.09	1.48±0.08	1.95±0.21	2.52±0.21
v_X	0.356±0.148	0.219±0.054	0.171±0.047	0.163±0.079	0.241±0.098
λ_X	2.58±0.90	0.92 (NS)	1.21 (NS)	-0.402 (NS)	0.482 (NS)
R^2	0.987	0.993	0.992	0.964	0.979
$Y_{X/Rs}$	0.364	0.786	0.609	0.751	1.10
$Y_{X/Pr}$	0.170	0.816	1.00	0.742	1.08
G_m	10.74 ±1.75	12.50±0.99	12.48±1.56	11.58±1.28	11.86±1.28
v_G	0.818±0.498	0.821±0.203	0.738±0.252	0.702±0.230	0.885±0.355
λ_G	1.93 (NS)	2.07±1.97	1.74 (NS)	1.01 (NS)	1.65 (NS)
R^2	0.948	0.990	0.980	0.982	0.978
$Y_{G/Rs}$	3.78	4.97	4.90	4.31	4.63
$Y_{G/Pr}$	11.07	5.16	8.08	4.25	4.56

Figure 1

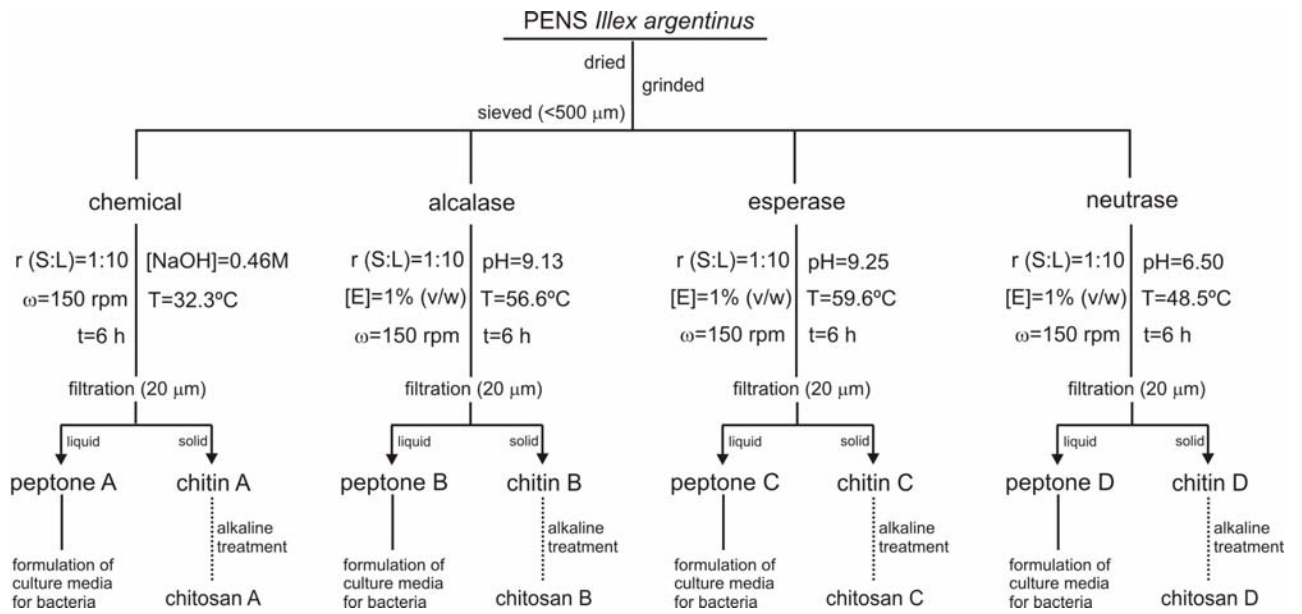

Figure 2

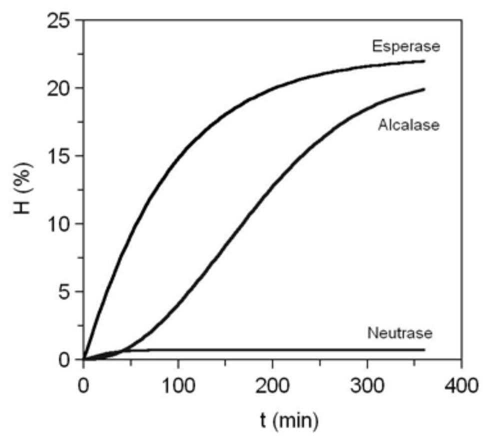

Figure 3

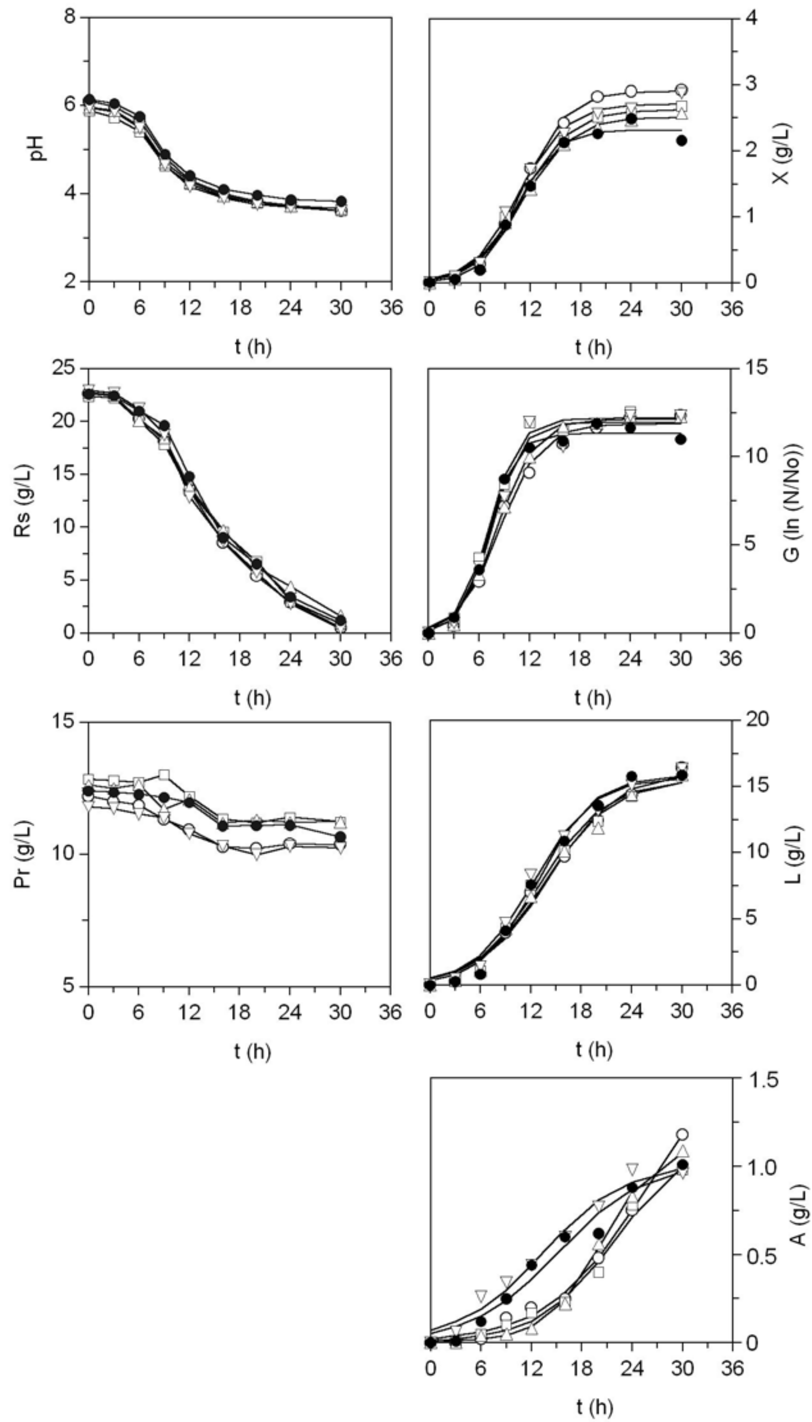

Figure 4

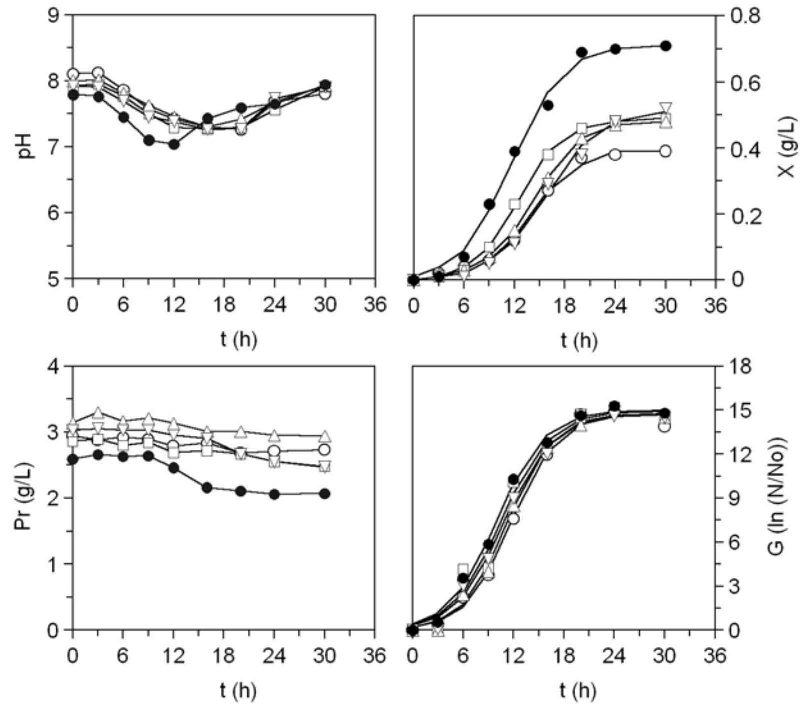

Figure 5

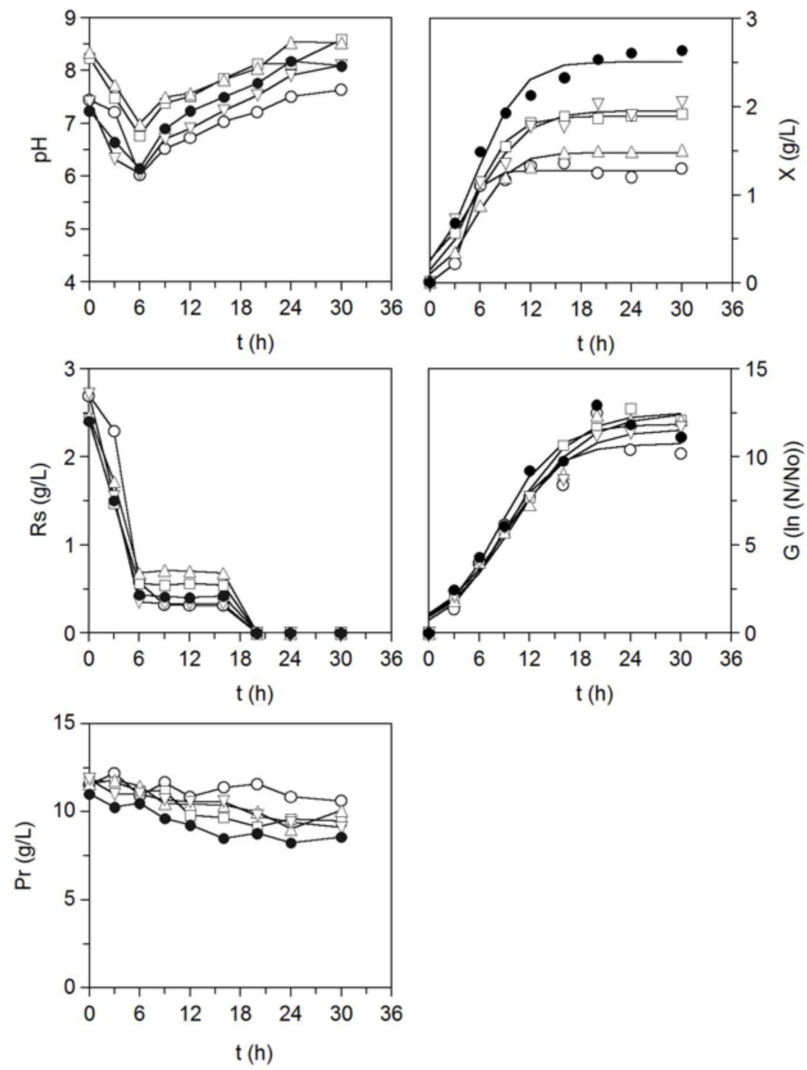

Table A. Composition of culture media for lactic acid bacteria (g/L).

INGREDIENTS	Medium A	Medium B	Medium C	Medium D	MRS
Glucose	20	20	20	20	20
Yeast extract	4	4	4	4	4
Sodium acetate	5	5	5	5	5
Ammonium citrate	2	2	2	2	2
K_2HPO_4	2	2	2	2	2
$MgSO_4$	0.2	0.2	0.2	0.2	0.2
$MnSO_4$	0.05	0.05	0.05	0.05	0.05
Tween 80	1	1	1	1	1
Meat extract	-	-	-	-	8
Bactopeptone	-	-	-	-	10
Peptones from *I.argentinus* pen as protein-Lowry	10	10	10	10	-

Medium A: formulated with Peptone A.
Medium B: formulated with Peptone B.
Medium C: formulated with Peptone C.
Medium D: formulated with Peptone D.

Table B. Composition of culture media for marine probiotic bacteria (g/L).

INGREDIENTS	Medium A	Medium B	Medium C	Medium D	MM
Ferric citrate	-	-	-	-	0.10
Sodium chloride	-	-	-	-	19.45
Magnesium chloride	-	-	-	-	5.90
Sodium sulphate	-	-	-	-	3.24
Calcium chloride	-	-	-	-	1.80
Potassium chloride	-	-	-	-	0.55
Sodium bicarbonate	-	-	-	-	0.16
Potassium bromide	-	-	-	-	0.08
Strontium chloride	-	-	-	-	34.0 mg
Boric acid	-	-	-	-	22.0 mg
Sodium silicate	-	-	-	-	4.0 mg
Sodium fluoride	-	-	-	-	2.4 mg
Ammonium nitrate	-	-	-	-	1.6 mg
Disodium phosphate	-	-	-	-	8.0 mg
Yeast extract	1.0	1.0	1.0	1.0	1.0
Peptone	-	-	-	-	5.0
Peptones from *I.argentinus* pen as protein-Lowry	2.6	2.6	2.6	2.6	-
Sea water (L)*	1	1	1	1	-
Distilled water (L)**	-	-	-	-	1

Medium A: formulated with Peptone A.
Medium B: formulated with Peptone B.
Medium C: formulated with Peptone C.
Medium D: formulated with Peptone D.
*Volume of filtrated and sterilized sea water needed for residual media preparation.
**Volume of distilled water needed for commercial medium preparation.

Table C. Composition of culture media for Gram (+) bacteria (g/L).

INGREDIENTS	Medium A	Medium B	Medium C	Medium D	TSB
Glucose	2.5	2.5	2.5	2.5	2.5
K_2HPO_4	2.5	2.5	2.5	2.5	2.5
NaCl	5	5	5	5	5
Casitone	-	-	-	-	17
Soy peptone	-	-	-	-	3
Peptones from *I.argentinus* pen as protein-Lowry	11	11	11	11	-

Medium A: formulated with Peptone A.
Medium B: formulated with Peptone B.
Medium C: formulated with Peptone C.
Medium D: formulated with Peptone D.