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# Production of Marine Probiotic Bacteria in a Cost-Effective Marine Media Based on Peptones Obtained from Discarded Fish By-Products

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**Abstract:** The industrial production of marine bacteria with probiotic properties is limited by the excessive cost of the culture media adequate for their growth. The present work aimed to study the suitability of 30 marine media formulated with nitrogen sources (fish peptones) from different fish discards and seawater, for the growth of two marine probiotic bacteria (MPB), namely *Phaeobacter* sp. and *Pseudomonas fluorescens*. These fish peptones were produced from several discarded fish and by-products (heads, skins, and whole individuals of megrim, mackerel, gurnard, hake, etc.). In all cultivations, no significant differences were found on cell viability when the microorganism grew on commercial or alternative media. In relation to the biomass production, the growth of *Phaeobacter* sp. on waste media was commonly similar or a 20% lower than observed in the control cultures. For *P. fluorescens*, various peptones (skin peptones of pouting and blue whiting) showed even higher productive ability than commercial peptones. An initial economical evaluation revealed that low-cost media reduced until 120 times the cost of production of MPB.

**Keywords:** fish discards valorization; marine probiotics bacteria production; fish peptones; low-cost marine media; sustainability; logistic equation

## 1. Introduction

In the last two decades, intensive investigation dealing with the search and application of probiotic bacteria in aquaculture has been extensively reported [1–4]. The problems associated with the extensive mortalities produced in the first larvae stages of fish farming and the restrictive legislation in the use of antibiotics have demanded new alternatives based on, for instance, the use of lactic acid bacteria [5,6] or marine bacteria [7–9] with remarkable probiotic capacities against the lethal microorganisms present in pisciculture diseases. Different marine probiotic bacteria (MPB) belong to the *Proteobacteria* phylum as *Shewanella*, *Pseudomonas*, and *Phaeobacter* genera have shown excellent behaviors to improve the growth of fish cultures, increasing the activity of gastrointestinal microbiota and the immune responses of fish [10–15]. Among MPB, *Pseudomonas fluorescens* and *Phaeobacter* sp. have demonstrated skills of ecological competitiveness against fish pathogen bacteria [13–16] and they also enhanced larvae survival when they were added to turbot, oyster and salmon hatcheries [10,16–18]. Although the use of

MPB has not yet been legalized in Europe, the need for solutions to avoid the application of antibiotics is increasing the pressure to commercialize effective bacteria to reduce fish farming mortalities.

In this context, the formulations of the media for the cultivation of bacteria are commonly rich in a source of organic nitrogen as free amino acids, protein hydrolysates, and/or peptones from different origins and commercial denominations: Bactopectone, tryptone, meat extract, etc. One of the most specific broths is the marine medium (MM) especially designed for the culture of bacteria isolated from marine environments, and commonly employed in the growth of MPB [11,14]. It is composed of multiple mineral salts, simulating seawater, a low concentration of yeast extract, and a generic peptone.

Nevertheless, the application of these marine probiotics to aquaculture plants and hatcheries, when they can be marketed, will require the production of massive biomasses and viable cells at the industrial scale. However, marine medium and even the commercial peptones are too expensive, and the development of a cost-effective medium is fundamental for a continuous and sustainable supply of MPB. Fish wastes could be an interesting source of cheap and efficient peptones, the application of which has resulted in excellent growth yields of an extensive number of bacterial genera [19,20].

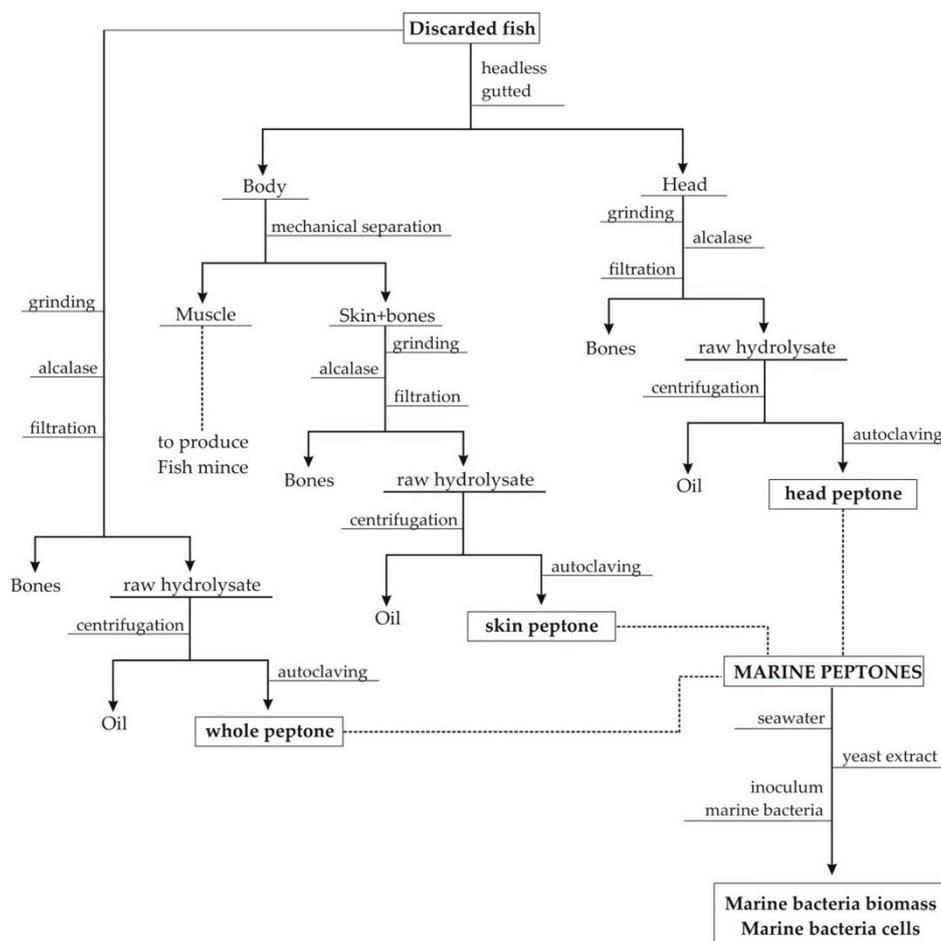
On the other hand, current European Union fishery policy is aimed at gradually eliminating fish discards. It forces fishing vessels to land all catches of regulated commercial species. The unwanted catches landed that cannot be directly sold for human consumption, due to the lack of a market, are considered as by-products [21]. As a result, the Landing Obligation (LO) policy will lead to an increasing amount of fish in European ports. While a fraction of this biomass may be suitable for the production of processed human food products, undersized and low-quality individuals require alternative plans [22,23]. Blue whiting, mackerel, megrim, gurnard, pouting, hake, Atlantic horse mackerel, hake, boarfish, and red scorpionfish are some of the most relevant, in tons, fish species landed in European ports but also the most discarded by fishing fleets [23], due to multiple motives: (a) Lack of quota, (b) species with low commercial value, or (c) being below legal sizes when are captured. One of the main challenges that faces the landing and valorize obligation imposed by the EU regulation No1380/2013, under the framework of the Common Fisheries Policy (CFP), is finding alternatives that help the fishery industry to overcome the costs associated with food-waste processing.

The present work investigated the potential of 30 peptones, obtained by enzymatic processing of whole individuals and head and skin by-products (obtained from fish mince production) of discarded fish generated from fishing activities, as a complex nitrogen source for the growth of *Phaeobacter* sp. and *P. fluorescens*. The kinetics of bacterial growth were perfectly modelled by a sigmoid equation formulated with parameters of clear biological meaning. Finally, a simple economical assessment was performed to corroborate the exceptional impact of the sustainable bioprocess proposed here.

## 2. Materials and Methods

### 2.1. Preparation of Peptones from Fish Discard By-Products

The selected fish discards (captured in Atlantic North Ocean by Galician fishing fleets): grenadier (Gr, *Macrourus* sp.), megrim (Me, *Lepidorhombus boscii*), European hake (Ha, *Merluccius merluccius*), boarfish (Bo, *Capros aper*), Atlantic horse mackerel (AHM, *Trachurus trachurus*), blue whiting (BW, *Micromesistius poutassou*), mackerel (Ma, *Scomber scombrus*), red scorpionfish (RS, *Scorpaena scrofa*), pouting (Po, *Trisoreptus luscus*), and gurnard (Gu, *Trigla* spp.), were separated, on board, from commercial species and stored in ice. In port, the species were processed in the same day of landing. One portion of discarded fish was manually gutted and headed, and meat mince was mechanically separated from bones and skin using a bone separator (Baader 694, Lübeck, Germany). This fish mince was frozen in blocks for the later preparation of burgers, nuggets, and fingers [23,24]. The mixture of skins with bones (Sk), heads (He), and another portion of whole individuals (Wh) that were discarded was used as substrates for the production of fish peptones (Figure 1).



**Figure 1.** Flowchart of steps involved for the production of peptones from the skin, head, and whole individuals of discarded fish by fishing fleets.

He, Sk, and Wh were ground in a meat grinder and stored at  $-18\text{ }^{\circ}\text{C}$ . Enzymatic hydrolysis of these materials was performed in a controlled pH-Stat system with a 5-L glass-reactor (mixing 1 kg of milled discards with 2 L of distilled water, (S:L) ratio of 1:2  $w/v$ ). Alcalase 2.4 L (Novozymes, Nordisk, Bagsvaerd, Denmark) was the commercial protease applied at 1% ( $v/w$ ), pH8.65, 200 rpm of agitation, and  $60\text{ }^{\circ}\text{C}$  for 4 h [25–27]. Bones were removed from raw hydrolysates by filtering and the liquid fraction was centrifuged ( $15,000\times g/20\text{ min}$ ) to separate oils and hydrolysates. The marine peptones were obtained after autoclaving ( $101\text{ }^{\circ}\text{C}/60\text{ min}$ ) and centrifuging these hydrolysates. A scheme of this procedure is shown in Figure 1. The basic composition of peptones is summarized in Table S1.

## 2.2. Microbiological Methods, Culture Media, and Analytical Determinations

The marine probiotic bacteria evaluated were *Phaeobacter* sp. DIFR 27–4 (Ph, called *Roseobacter* sp. before) and *Pseudomonas fluorescens* DIFR AH-2 (Pf). Ph and Pf were kindly provided by Dr. Lone Gram (DTU Aqua, Lyngby, Denmark). Stock cultures were stored at  $-80\text{ }^{\circ}\text{C}$  in MM (Difco, Becton, Dickinson and Company, Sparks, MD, USA) with 25% glycerol. Inocula (1%  $w/v$ ) consisted of cellular suspensions from 12–16 h cultures on MM. The composition of the culture media is shown in Table S2. The protein concentration in the media formulated with fish peptones was established by replacing the Lowry protein level in the peptone (2.6 g/L) present in MM. Mineral salts of this commercial broth were replaced by filtered and sterilized seawater. Thus, in alternative media, the fish peptones were mixed with seawater and 1 g/L of yeast extract (this was the only commercial ingredient added). Fermentations were carried out in triplicate using 300 mL Erlenmeyer flasks with 150 mL of medium at  $22\text{ }^{\circ}\text{C}$  and 200 rpm of orbital shaking. In all cases, the initial pH was adjusted to 7.5 with NaOH (5 N)

and the media was sterilized separately at 121 °C for 15 min. The basic composition of fish peptones was determined in duplicate, as follows: (1) Reducing sugars (RS) by 3,5-dinitrosalicylic reaction [28], (2) total soluble proteins (Pr) using the Lowry method [29], and (3) total sugars (TS) by Dubois et al.'s protocol [30].

### 2.3. Bacterial Sampling and Biomass and Cell Analysis

Samples from each culture in alternative and control media were taken at predetermined times (3, 6, 9, 12, 16, 20, 24, and 30 h) and divided in two aliquots. The first aliquot was used for quantifying viable cells by means of a plate count technique on MM agar. Serial 10-fold dilutions were prepared in peptone-buffered solutions, and 0.1 mL samples were plated in triplicate, incubated at 22 °C 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  $3270\times g$  for 15 min, from which the supernatant was used for determining the protein (Pr, Lowry method) consumption and the sediment was washed and resuspended in distilled water at an appropriate dilution to measure the optical density (OD) at 700 nm ( $A_{700}$ ). Then, the dry weight was estimated from a calibration curve ( $A_{700}$  vs. dry weight).

### 2.4. Mathematical Equations for Cultures Modelling

The growth of marine bacteria, biomass production ( $X$ ), and cell formation ( $G$ ) were predicted by the logistic equation [31]:

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

Additionally, other parameters from Equation (1) were also calculated with the purpose of evaluating all the characteristics phases of the sigmoid growth [31]:

$$\mu_P = \frac{4v_P}{P_m} \quad (2)$$

$$\tau_P = \lambda_P + \frac{2}{\mu_P} \quad (3)$$

$$t_{mP} = \tau_P + \frac{P_m}{2v_P} \quad (4)$$

where,  $P$  is the growth determined ( $X$  for biomass or  $G$  for viable cells);  $t$  is the time of culture (h);  $P_m$  is the maximum growth (g/L for  $X$  and dimensionless for  $G$ );  $v_P$  is the maximum growth rate ( $\text{g L}^{-1} \text{h}^{-1}$  for  $X$  and  $\text{h}^{-1}$  for  $G$ );  $\lambda_P$  is the growth lag phase (h);  $\mu_P$  is the specific maximum growth rate ( $\text{h}^{-1}$ );  $\tau_P$  is the time required to achieve half of the maximum growth (h); and  $t_{mP}$  is the time required to reach the maximum growth (h). In addition, the yields of growth on protein uptakes ( $Y_P/Y_{Pr}$ ) were also determined.

### 2.5. Economical Assessment of MPB Growth Costs

Based on the market prices of the MM ingredients and the values of  $X_m$  and  $G_m$  summarized in Tables 1 and 2 and Tables S3 and S4, the costs of biomass (in €/g) and cells (in €/cell) production were calculated for the cultures with both bacteria in all media. In these calculations, the costs of the production of fish peptones (energy and reagents) were not included. These costs are highly dependent on the production scale of peptones (relatively higher on a smaller level of production) and difficult to determine on a laboratory scale. The present strategy of the valorization of heads and skins to produce fish peptones is a complementary alternative associated to the recovery of fish mince from fish discard biomasses that have to be landed under the LO normative. For a complete economical evaluation of such MPB, the process of fish mince production should also be incorporated in a further study.





## 2.6. Numerical and Statistical Analyses

Fitting procedures and parametric estimations calculated from the results were carried out by minimizing 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 the consistency of mathematical models (Fisher’s *F* test) and residual analysis (Durbin–Watson test) were evaluated by “SolverAid” macro (Levie’s Excellaneous website: <http://www.bowdoin.edu/~rdelevie/excellaneous>).

## 3. Results

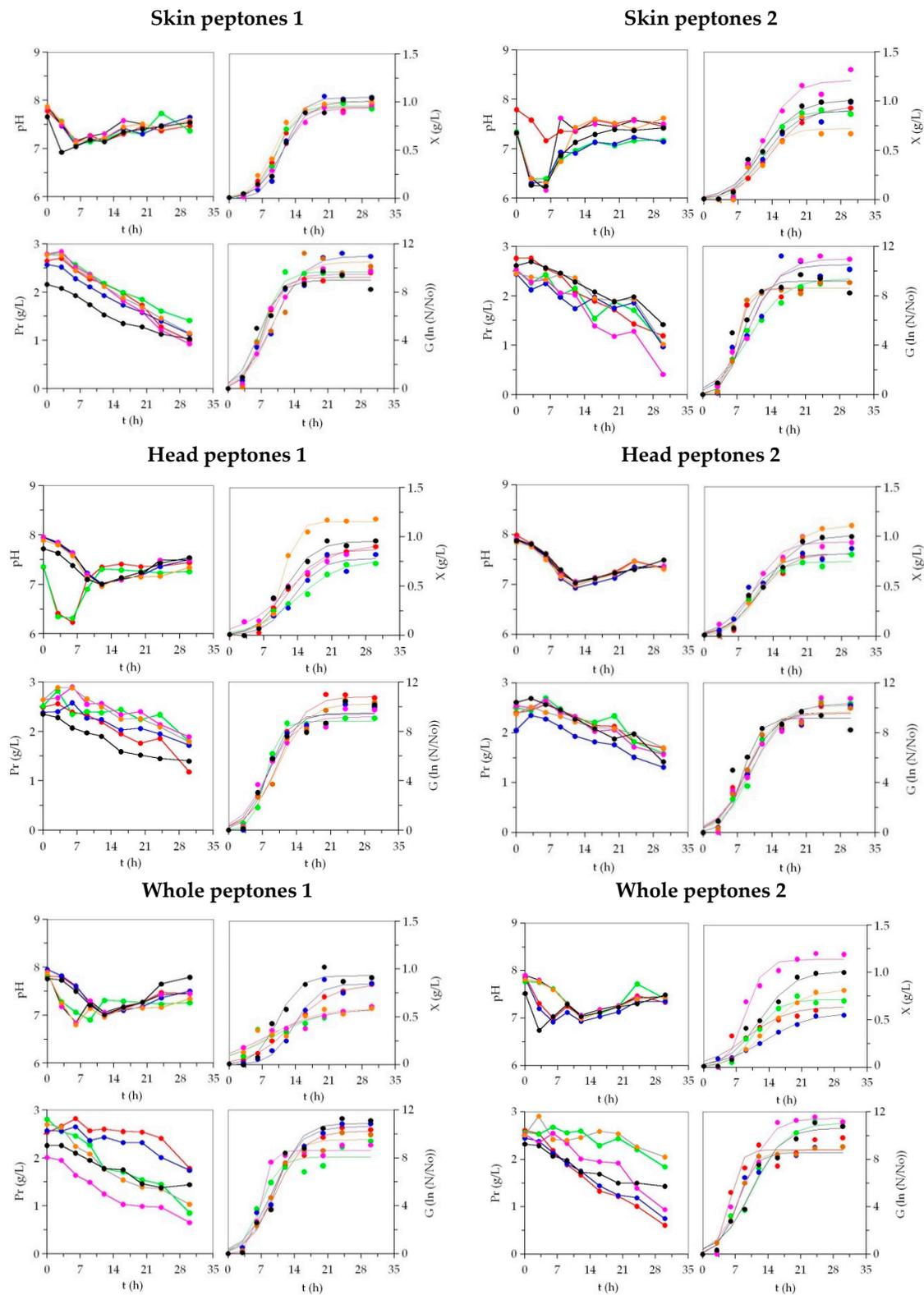
Thirty fish peptones were produced by enzymatic hydrolysis of whole specimens of discarded fish and from by-products of these discards generated after fish mince recovery. They were prepared at a concentration of 2.6 g/L of Lowry protein (similarly to the standard level in MM) and the results were compared to the cultivations performed in commercial medium.

Table S1 summarizes the basic chemical composition of the fish peptones. The content of total soluble protein varied from 33 to 45 g/L for Sk, 28 to 40 g/L for He, and 36 to 54 g/L for Wh. The peptones also contained small amounts of total (0.45–1.61 g/L) and reducing sugars (0.09–0.45 g/L) due to the initial presence of fish muscle (tissue that accumulates a reserve of sugars) in the substrates employed for the production of such peptones. Taking into account that the new marine media included as maximum 10% of the final volume with fish peptones, the final concentrations of total and reducing sugars in these media were insignificant and always lower than 0.05 and 0.16 g/L, respectively.

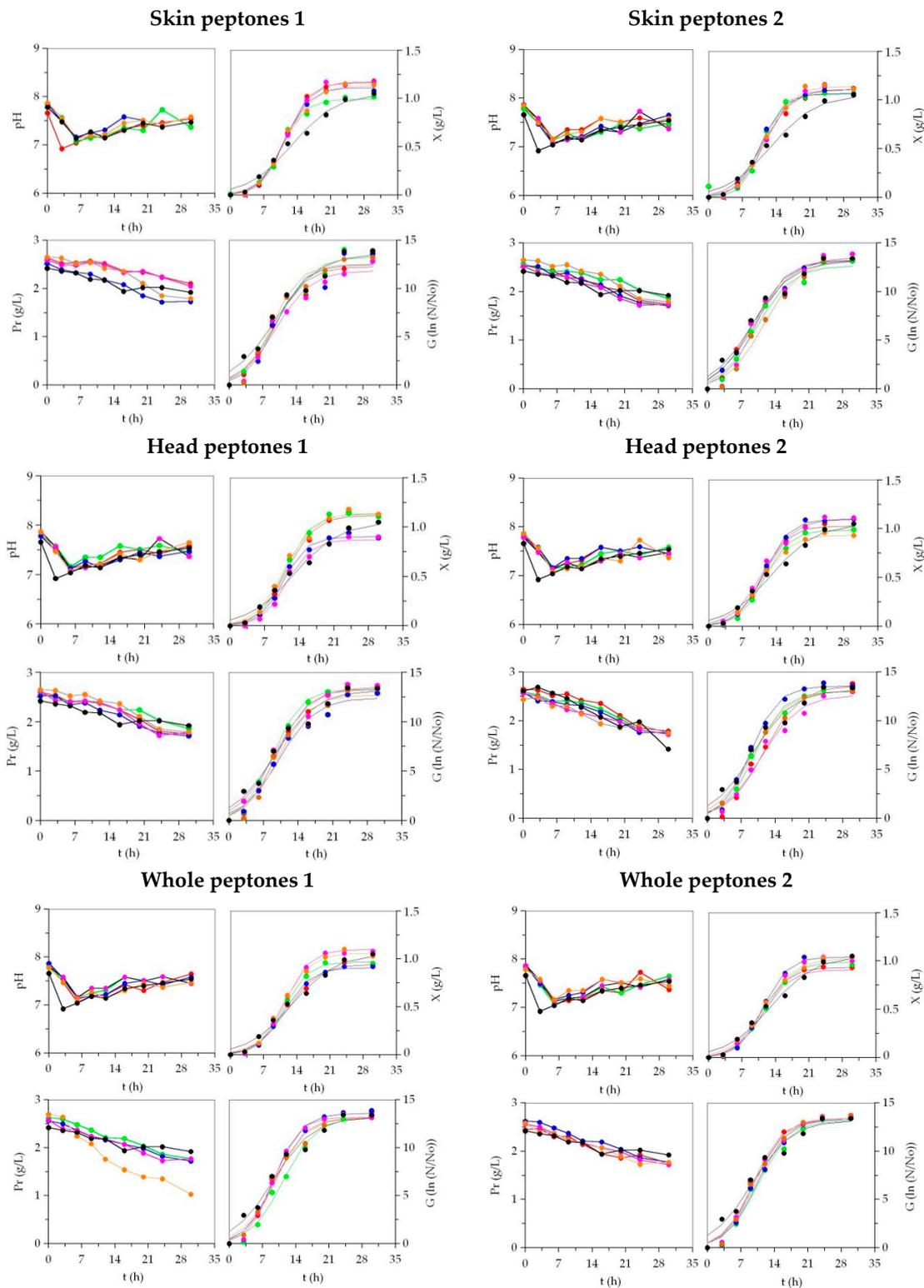
*Phaeobacter* sp. and *P. fluorescens* grew perfectly in all media tested (Figures 2 and 3). The time-course of pH in the low-cost media generally followed similar patterns, with little exceptions, to those defined by MM. The experimental data of the growth, by optical density and plate-count, were in all cases mathematically modelled using the logistic Equation (1). The agreement between the experimental and predicted data was very good, with a goodness of fit that varied from 0.917 to 0.999 (Tables 1 and 2 and Tables S3 and S4). In addition, the consistency of the logistic equation was corroborated for all the fittings by the F-Fisher test ( $p < 0.005$ ). The numerical parameters from such an equation were statistically significant (t-Student test) except in 19 cases of 720, mainly lag-phases ( $\lambda_x$  and  $\lambda_G$ ), where latencies were not clearly observed nor numerically identified.

In *Phaeobacter* sp. cultures (Tables 1 and 2), higher  $X_m$  values were mostly obtained in peptones of Sk than in peptones of He and Wh, but they were similar to the control outcomes (with exception of SK\_AHM). The lowest maximum biomass production was found in the peptones of Wh. Nevertheless, the largest values were reached in Sk\_Bo (1.21 g/L), He\_Gu (1.16 g/L), and Wh\_Bo (1.14 g/L), the first two being significantly more productive than MM ( $p < 0.05$ ). The growth quantified by viable cell production followed a similar response in alternative peptones: The Sk origin was slightly most valuable than He and Wh, and the best options were Wh\_Bo, Sk\_Bo, and Sk\_Gu. The growth rate parameters ( $v_x$ ,  $v_G$ ,  $\mu_x$  and  $\mu_G$ ) were higher in some discard-based peptones than in MM, but the differences were not significant ( $p > 0.05$ ). These findings were in agreement with the time-dependent parameters ( $\lambda_x$ ,  $\lambda_G$ ,  $t_{mx}$ ,  $t_{mG}$ ,  $\tau_x$ , and  $\tau_G$ ) that show similar statistical values between all media.

The maximum biomass production of *P. fluorescens* in media containing Sk\_peptones was also higher than in the rest of the peptones. Peptones from the skins of BW and Po revealed the best results whereas peptones from the heads of Bo and Gr led to the lowest dry weights. The numerical data in both pairs of peptones were statistically different in comparison to that predicted for MM ( $p > 0.05$ ). Faster biomass production ( $v_x$  and  $\mu_x$ ) and shorter time-dependent parameters ( $t_{mX}$  and  $\tau_x$ ) were reached in MM (Tables S3 and S4). Nevertheless, the improvements of the kinetic parameters in the commercial medium compared with most of the low-cost media were not significant. The results of viable cell growth for *P. fluorescens* in the different media were visually almost indistinguishable and parametrically similar between cultures. No significant differences between media were found for all the parameters that characterize such growth determinations.



**Figure 2.** Culture kinetics of *Phaeobacter* sp. in marine media based on marine peptones from discarded fish and by-products. Peptones 1: ●: BW; ●: RS; ●: Ma; ●: Po; ●: Gu; ●: MM1. Peptones 2: ●: Gr; ●: Me; ●: Ha; ●: Bo; ●: AHM; ●: MM2. Experimental data of biomass ( $X$ ) and viable cells ( $G$ ) were fitted to the logistic equation. Protein ( $Pr$ ) uptakes and pH are also shown. The confidence intervals of the experimental data (for two replicates) were in all cases less than 20% of the experimental mean values and omitted for clarity.



**Figure 3.** Culture kinetics of *P. fluorescens* in marine media based on marine peptones from discarded fish and by-products. Peptones 1: ●: BW; ●: RS; ●: Ma; ●: Po; ●: Gu; ●: MM1. Peptones 2: ●: Gr; ●: Me; ●: Ha; ●: Bo; ●: AHM; ●: MM2. Experimental data of biomass (X) and viable cells (G) were fitted to the logistic equation. Protein (Pr) uptakes and pH were also shown. The confidence intervals of experimental data (for two replicates) were in all cases less than 20% of the experimental mean values and omitted for clarity.

The production yields for *Phaeobacter* biomass ( $Y_X/Y_{Pr}$ ) were globally larger in He\_peptones than Sk\_peptones, Wh\_peptones, and MM, but the best yield was found in Wh\_AHM. However, for Pf, those ratios of biomass production as a function of the protein uptake showed larger values in Sk\_peptones, with BW, Po, and Me being the most efficient substrates. In the case of viable cells of MPB ( $Y_G/Y_{Pr}$ ), the generic trends were similar to those described for  $Y_X/Y_{Pr}$ : He\_peptones and Sk\_peptones as the best productive source of organic nitrogen for *Phaeobacter* sp. and *P. fluorescens*, respectively. Sk\_Me, SK\_BW, and Sk\_Po in *Phaeobacter* sp. and Sk\_Gu in *P. fluorescens* were the most efficient media in the consumption of proteins.

Table S5 shows the costs of the production of MPB biomass and viable cells in the new marine media (based on fish peptones and seawater) and in the corresponding commercial MM. Compared against the control, the decrease of biomass and cell growth costs for *Phaeobacter* sp. ranged from 68–120 times and 81–107-fold, respectively, depending on the fish peptone used (with a higher reduction in Sk\_Bo and He\_Gu). In the case of *P. fluorescens*, these reductions in relation to MM production were in the interval 83–111 times for  $X_m$  and 88–101 times for  $G_m$  (with a lower decrease in He\_Bo and He\_Gr).

#### 4. Discussion

In the current study we completed the total valorization of wastes (heads and skins with bones) generated in the previous manual and mechanical separation of fish mince useful for the elaboration of human foods [24]. This sustainable strategy was also extended to the whole individuals of the same discarded species, which, in some situations, are not allowed to be introduced in the preparation of products for human consumption [23].

Our results indicated that the nutritive formulations based on the mixture of peptones obtained from fish discards' hydrolysis and seawater are cost-effective alternatives to produce marine probiotic bacteria at a large scale. Most of them were perfect substitutes of commercial marine medium for both bacteria studied. The maximum production of biomass and cells by Ph on the cited media was similar to those obtained in media containing peptones extracted from yellowfin tuna wastes and more than 20% higher than the production found using rainbow trout, swordfish, and squid by-products [32,33]. In the case of Pf, the present results also improved the production observed in those referenced organic nitrogen sources [32,33]: The values of maximum biomass in most of the alternative media (such as those formulated here with Sk\_BW, Sk\_Po, Sk\_Gu, and He\_Po) were 30% superior to the best results reported for peptones from squid and trout viscera. Additionally, tuna and trout waste materials were demonstrated to be an adequate source of nitrogen for the production of alkaline proteases by two *Vibrio* species [34]. This genus and the metabolites were not studied here and could be an interesting potential application of peptones from discarded fish in further works.

In terms of processing, the recovery of protein sources from fish wastes by enzyme proteolysis is a well-documented alternative [35,36]. One of the cheapest approaches is using the endogenous enzymes present in the pancreas or pyloric cecum (autohydrolysis step) when fish viscera residues are valorized [37–39]. The peptones thus obtained have been useful for the production of bacteriocins (nisin and pediocin) by *Lactococcus lactis* and *Pediococcus acidilactici* [37,40]. However, the difficulty in controlling the process of biocatalysis with the corresponding variability on the kind and size of peptides generated advises the application of exogenous enzymes. Between commercial proteases, alcalase (an endoprotease) is one of the most valuable for the digestion of fully fish residues to yield high degrees of hydrolysis and in vitro digestibility, and to tailor the molecular weight of the peptides [32,36,41–43]. For instance, hydrolysates of tuna head and cod viscera obtained by alcalase were adequate for lactic acid bacteria production [19,44]. Nevertheless, few studies of marine bacteria on fish protein hydrolysates have been conducted. Biomasses of *Vibrio anguillarum*, *Roseobacter* sp., *Carnobacterium divergens*, and *Aeromonas salmonicida* were successfully produced in peptones from fish by-products and cephalopod effluents [20,37,45]. The low-cost marine media developed here,

including filtered seawater, yeast extract, and peptones from hydrolysates of discarded species and by-products, has been unexplored to date.

Finally, we must indicate that the proposed evaluation of cultures by means of the kinetic parameters obtained from Equation (1) was in all cases successful since the experimental data were perfectly simulated and the parameters defined all the sigmoid growth phases [31]. The logistic equation is a well-known mathematical resource extensively utilized in different fields, such as animal growth [46,47], predictive microbiology [48], microbial metabolite productions [49], and analysis of DNA sequencing [50], among others. The present economic decreasing production costs for biomass and cell formation of MPB were in agreement with values previously reported for protein-rich effluents from chitin purification [32]. The very low cost of MPB growth on alternative marine media may suppose a valuable tool for the sustainable management of discarded fish residues and for the massive production of viable probiotics adequate for fighting infections in aquaculture fish diseases.

## 5. Conclusions

This work showed that a cost-effective media formulated with peptones obtained by the enzyme hydrolysis of fish discards (head, skins, whole individuals), filtered seawater, and a very low concentration of yeast extract can be an excellent substitute of the commercial marine medium to produce marine probiotic bacteria, such as *Phaeobacter* sp. and *Pseudomonas fluorescens*. For both bacteria, viable cellular productions were almost always, at least, similar to those found in MM. The biomass kinetics (quantified as dry weight) of Pf and Ph showed similar or slight differences between the low-cost and control media. In economic terms, the alternative media led to a huge reduction of marine probiotic bacteria growth costs, between 68- and 12-fold depending on the strain and variable evaluated, in comparison with those found in commercial MM.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/2076-2607/8/8/1121/s1>, Table S1: Basic biochemical composition of fish peptones, Table S2: Composition of culture media for marine probiotic bacteria, Table S3: Parameter kinetics of Pf cultures, Table S4: Continuation of Table S3, Table S5: Cost of production of biomass and bacterial cells.

**Author Contributions:** Conceptualization, J.A.V.; Data curation, J.A.V.; Formal analysis, J.A.V.; Funding acquisition, J.A.V., A.M.G., and A.C.F.; Investigation, J.A.V., A.D., A.M. and M.N.; Methodology, J.A.V., A.D., A.M. and M.N.; Project administration, J.A.V. and A.M.G.; Supervision, J.A.V.; Writing—original draft, J.A.V.; Writing—review and editing, J.A.V., A.M.G., J.A., and A.C.F. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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